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

II. Pathway and Turgor Sensitivity

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ABSTRACT

Phloem import and unloading in perfused bean (*Phaseolus vulgaris* L.) seed coats were investigated using steady-state labeling. Though photosynthate import and unloading were significantly reduced by perfusion, measurements of photosynthate fluxes in perfused seed coats proved useful for the study of unloading mechanisms *in vivo*. Phloem import was stimulated by lowered seed coat cell turgor, as demonstrated by an increase in tracer and sucrose import to seed coats perfused with high concentrations of an osmoticum. The partitioning of photosynthates between retention in the seed coat and release to the perfusion solution also was turgor sensitive; increases in seed coat cell turgor stimulated photosynthate release to the apoplast at the expense of photosynthate retention within the seed coat. There was no evidence of a turgor-sensitive sucrose uptake mechanism in perfused seed coats. Thus, the turgor sensitivity of photosynthate partitioning within perfused seed coats was consistent with a turgor-sensitive efflux control mechanism. Measurements of tracer equilibration and sugar partitioning in perfused seed coats provided strong evidence for symplastic phloem unloading in seed coats.

The empty seed coat technique permits the analysis of photosynthates released from maternal seed tissues following the surgical removal of the embryo and the addition of a trapping solution or agar (12, 18, 22). Sucrose is quantitatively the most important material eluted from perfused seed coats of species studied to date (12, 17, 18, 21, 22).

The efflux of pulse-labeled photosynthates and unlabeled sugars (and amino acids) from seed coats attached to the plant via the funiculus is enhanced by perfusion with solutions containing high concentrations of an osmoticum (1, 6, 10, 23–26). The mass flow hypothesis of phloem transport predicts that lowering the turgor pressure of sieve elements within sink tissues will enhance phloem import into sinks (7). Osmoticum concentration does not influence seed coat membrane permeability (determined by [^{14}C]mannitol-uptake experiments) (14). Thus, the enhancement of photosynthate efflux from attached seed coats by high concentrations of osmoticum has been attributed to the stimulation of phloem import by low cell turgor (1, 6, 9, 23–26).

Photosynthate efflux from seed coats detached at the funiculus is enhanced by lower osmoticum concentrations in the perfusion solution (6, 12–14, 25, 26). The conflicting results obtained with detached *versus* attached seed coats have been explained by the hypothesis that photosynthate efflux from attached, perfused seed coats is dominated by phloem import (stimulated by low cell turgor), whereas photosynthate efflux from detached, perfused seed coats is controlled by a mechanism acting on the efflux itself (6, 26). Low osmoticum concentrations have also been shown to increase photosynthate efflux from attached, perfused seed coats (1, 6, 9, 13, 14, 25, 26).

The effect of low osmoticum concentrations on efflux may be explained by either an increase in efflux through a turgor-sensitive carrier (the turgor homeostat hypothesis) (13, 14), or by a decrease in uptake by a turgor-sensitive apoplastic retrieval mechanism (25, 26). The turgor homeostat hypothesis is supported by ^{14}C-photosynthate efflux studies (13, 14). Turgor-sensitive sugar retrieval has been studied indirectly using labeled and unlabeled sugar efflux measurements, but turgor-sensitive sugar uptake was not measured in these experiments (25, 26). This study will attempt to distinguish between the apoplastic retrieval and the turgor homeostat hypotheses of photosynthate efflux regulation.

Because maternal and embryonic tissues are symplastically isolated in legume seeds, photosynthate must unload to the seed coat apoplast before uptake by the embryo (17, 21). Apparently there are sufficient plasmodesmata to accommodate symplastic transfer of photosynthate throughout the seed coat before it is unloaded into the apoplast in *Phaseolus vulgaris* L. (the symplastic unloading route hypothesis) (11). This hypothesis has also received support from empty seed coat experiments (6, 14) and studies of tracer kinetics (2, 15). An alternative to the symplastic unloading route hypothesis is that photosynthates are unloaded directly from the phloem into the apoplast, without passing through the seed coat symplast (the apoplastic unloading route hypothesis) (20). Although most evidence supports the symplastic unloading route hypothesis, there has been no definitive confirmation of this pathway.

This study will demonstrate quantitative methods for the analysis of turgor-sensitive photosynthate transport in per-

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fused seed coats, and will also attempt to distinguish the pathway of phloem unloading in seed coats. Steady-state labeling with $^{14}CO_2$ will be used to simplify analysis of tracer fluxes within seed coats (2). Disruption of phloem import and unloading by the empty seed coat technique has recently been reassessed (2, 10), and will be evaluated quantitatively in this study. To clarify discussion of photosynthate movement into and out of perfused seed coats, we will use the following terms, as proposed by Grusak and Minchin (6); *phloem import*: the movement of photosynthates into the seed coat via the phloem; *phloem unloading*: the movement of assimilates from the phloem to the apoplast *or* the symplast of the seed coat; and *seed coat unloading*: the movement of photosynthates from the seed coat symplast to the apoplast.

MATERIALS AND METHODS

Plant Preparation, Steady-State Labeling, and Seed Coat Perfusion

A determinate variety of bean (*Phaseolus vulgaris* cv Redkloud) was grown in the greenhouse, and plants were prepared for steady-state labeling and perfusion experiments 15 to 25 DAF. Plants were labeled in a forced-draft hood as described by Ellis *et al.* (2). In summary, steady-state labeling was initiated between 8:00 and 9:30 AM by sealing the entire top leaf in an assimilation chamber about 9 min after beginning the light period. A 1000 W metal halide lamp was used to provide a PPFD of 1000 μmol photons m^{-2} s^{-1} to the labeled leaf. Approximately 20 min after beginning steady-state labeling, "empty" seed coats were prepared by the following protocol: (a) about 45% (by fresh weight) of the distal sides of two adjacent seeds were surgically removed, leaving at least one intact seed on either side of the incision (2), (b) the surface of the incision was smeared with silicone stopcock grease, (c) the embryos of the cut seeds were lifted out of the seed coat in one piece using a weighing spatula, and (d) the attached seed coat "cups" were rapidly flushed with distilled water to remove any adhering cotyledon fragments. To determine the effect of funicular attachment on sugar efflux, one of the two empty seed coats was selected at random and detached from the pod by gently prying up the seed coat and breaking the funiculus. Funicular breakage was observable when it occurred, because detached seed coats moved slightly out of the incision in the pod wall, and were easily pulled out of the pod.

Perfusion was initiated within 5 min of empty seed coat preparation. Seed coats were perfused with a buffer consisting of 0.5 mM $CaCl_2$, 1.0 mM KCl, 5.0 mM Mes, pH 6.0 (NaOH), and containing various concentrations of mannitol as an osmoticum. A multichannel peristaltic pump (Cole-Parmer Co., Chicago, IL) transported perfusion solution into the seed coat through a tube (polyethylene, 0.60 mm o.d.) positioned near the bottom of the seed coat cup. Another channel of the pump removed perfusate from the seed coat through a tube positioned 1.5 mm below the edge of the incision. Solution was pumped from the seed coat more rapidly than solution entered, so that bubbles were formed in the tube leaving the seed coat. A fairly constant level of solution was maintained in the seed coat cup by this method.

The rate of perfusion was between 75 and 175 μL min^{-1} with a typical rate of 100 μL min^{-1}, and varied no more than 25% during an individual experiment. Twenty-minute samples of perfusate were collected using a fraction collector. The first 20-min sample was discarded, due to difficulty in establishing uniform flow rates during the perfusion set-up period. At the end of perfusion, seed coats were checked for attachment at the funiculus by pulling on them with forceps. Data from seed coats that were accidentally detached were similar to data from intentionally detached seed coats, but are not reported here.

Sugar and ^{14}C Analysis

Methods for the analysis of sugar and ^{14}C in tissues have been described (2). Perfusate sugar and ^{14}C were assayed directly by methods used previously for neutral column eluates (2). Direct analysis of perfusate sugar and ^{14}C content led to overestimation of perfusate sugar specific activity because nonsugar ^{14}C compounds were present in perfusates. Tracer data were normalized to a specific activity of 1.0 GBq $(mol\ C)^{-1}$ (2). The difference between sugar efflux from attached *versus* detached seed coats at each time interval was calculated for each experiment by subtracting the efflux of the detached seed coat from the efflux of the attached seed coat within each pod.

Size Adjustment for Perfused Seeds

To facilitate comparisons between intact and perfused seeds, a seed size correction factor based on the sugar content of the basal portion of cut seed coats (approximately 70% of intact seed coat sugar content) was used to compensate for the removal of a fraction of perfused seed coats. The seed coat sugar content correction factor produced corrected data that were 73% of values obtained by using the alternative adjustment of correcting for the proportion of seed fresh weight remaining after cutting the seed (approximately 55% of intact seed fresh weight). In the absence of an independent criterion for selection of a correction factor (sugar content or fresh weight), the sugar content correction factor was chosen to reduce the likelihood of overestimating values from perfused seed data.

Statistical Methods

Analysis of covariance (16, pp. 366–371) was used to quantify and adjust for plant-to-plant variability in ^{14}C and specific activity data. Analysis of covariance makes use of internal controls (or "covariates") that are uninfluenced by treatments to provide a measure of the inherent variability that exists in a given parameter from experiment to experiment. In the present study, covariance analysis was used to determine whether there was a significant linear relationship between tracer data from the intact (covariates) and perfused (treatments) seeds within a pod. When there was a statistically significant linear relationship between measurements from intact and perfused seeds, the plant-to-plant variability quantified by intact seed measurements (controls) was used to adjust perfused seed data. The final products of covariance

analysis were adjusted treatment means, which were theoretically equivalent to the values that would be produced by the treatments had there been no variability from plant to plant. The equivalent ^{14}C fractions were used for both the intact and perfused seeds in a covariance analysis, and the intact seed coat neutral fraction was used as a covariate for perfusate ^{14}C data. Fisher's protected LSD (16, pp. 233–236) was used to determine whether there were statistically significant differences between treatment means, with or without the application of covariance adjustment.

Sucrose Uptake Experiments

In the morning (between 10:00 AM and 1:00 PM) seeds were removed from pods, and the seed coats excised by making a longitudinal incision around the seed coat through the hilum and funicular region. Excised seed coat pairs (one pair from each seed) were enclosed in cheesecloth bags and placed in beakers containing seed coat perfusion solution (see above). Mannitol concentrations in the solutions were varied to produce osmoticum treatments; these concentrations were kept constant throughout each experiment (rinses included).

Seed coats were pretreated in the sucrose-free perfusion solution for 1 h to simulate the conditions of perfusion experiments. They were then blotted and plunged into fresh perfusion solution containing 200 μM [U-^{14}C]sucrose with a specific activity of 4.59 GBq (mol C)$^{-1}$ (Sigma, St. Louis, MO). The sucrose concentration used for uptake was similar to the sucrose + glucose concentration of seed coat perfusates after 1 h of perfusion (100–200 μM).

Seed coats were removed from the uptake solution after defined time periods, quickly blotted, dipped in an unlabeled rinse solution, blotted again, and placed in a second unlabeled rinse solution for 5 min. Following the second rinse, seed coats were blotted and snap frozen in liquid nitrogen. All solutions (except the first rinse) were vigorously aerated during seed coat immersion.

Prior to ^{14}C determination, seed coats were quickly thawed, removed from the cheesecloth bags, and dried in an oven at 75°C. Dried seed coats were oxidized and counted as described previously for embryos (2). ^{14}C accumulation was approximately linear for the first 25 min of uptake. The 5 min rinse reduced the relatively large quantity of label in the apoplast without significant label efflux from the symplast. However, this short rinse did not completely remove apoplastic sucrose, since ^{14}C uptake could not be extrapolated to zero at zero time (data not shown). Sucrose uptake rates were calculated by subtracting the total seed coat ^{14}C content after a 5-min uptake period from the ^{14}C content after an 18-min uptake period. Uptake rate calculation from the difference in tracer accumulation at two different uptake periods eliminates the need for a complete apoplastic washout for the prediction of uptake into the symplast of a tissue.

RESULTS

Sugar Efflux from Perfused Seed Coats

The kinetics of sugar efflux from seed coats attached to the plant via the funiculus (attached-perfused treatment) were clearly different from those of seed coats detached from the funiculus (detached-perfused treatment); sugar efflux from detached-perfused seed coats declined rapidly over time (Fig. 1B), whereas efflux from attached-perfused seed coats decreased more slowly (Fig. 1A). The time course of detached seed coat sugar elution approximated a tissue washout, in that there was a continuous exponential decrease in efflux over time. The relatively slow decline in sugar efflux from attached-perfused seed coats was most likely due to the continued phloem import of sugar. Sugar efflux was significantly greater in attached seed coats than in detached seed coats between 2 and 8 h after the beginning of perfusion; before 2 and after 8 h of perfusion, sugar efflux was similar (Fig. 1C).

Osmoticum concentration did not affect the amount of sugar eluted over the full 12-h perfusion period in either attached or detached seed coats (Table I). In addition, osmoticum concentration had no statistically significant effect on the magnitude or slope of the sugar efflux time course between 2 and 8 h after beginning perfusion (not shown). However,

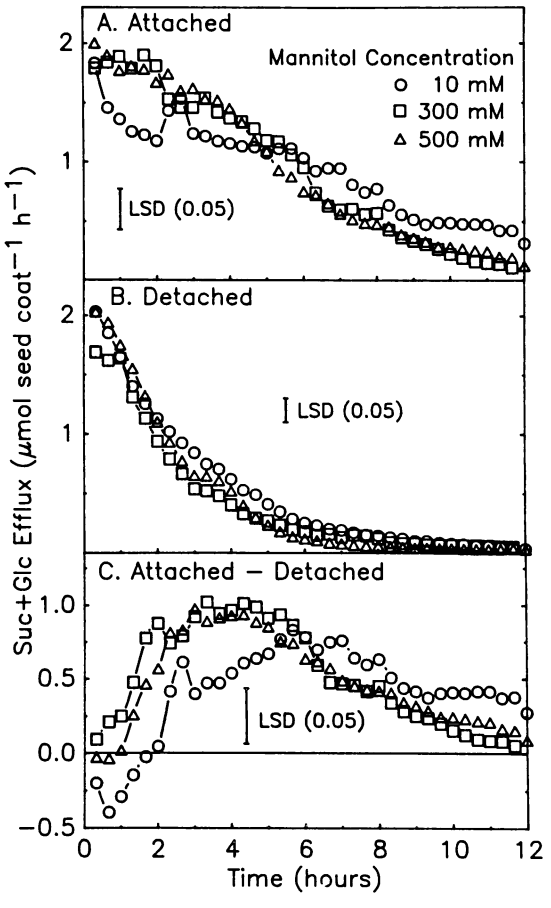

Figure 1. Sugar (sucrose + glucose) efflux from perfused seed coats. A, Efflux from attached-perfused seed coats; B, efflux from detached-perfused seed coats; C, difference between sugar efflux from attached *versus* detached seed coats. Data for each mannitol concentration are the mean of three experiments, and the LSD ($P = 0.05$) between the 12-h mean efflux rates is shown. No standard error data are presented. However, standard error tended to increase toward the end of the perfusion period. Zero time refers to the beginning of seed coat perfusion.

the amount of sugar remaining in attached seed coats after 12 h of perfusion was greater when higher concentrations of osmoticum were used (Table I). At the highest osmoticum concentration (500 mM), the amount of sugar remaining in attached-perfused seed coats was similar to the amount present in intact seed coats (Table I, values in parentheses). The sugar content of detached-perfused seed coats appeared to increase at higher concentrations of mannitol, but this effect was not statistically significant (Table I). After perfusion, detached seed coats contained less sugar than attached-perfused seed coats (Table I).

In attached-perfused seed coats, the amount of sugar eluted plus the amount retained after perfusion was greater than the amount present in intact seed coats at all osmoticum levels (Table I). This demonstrates net import of sugar into attached-perfused seed coats. Higher osmoticum concentrations enhanced the combined total of sugar eluted from, and remaining in, attached-perfused seed coats, but had no significant effect on detached-perfused seed coats (Table I). This indicates that high osmoticum concentrations enhanced net sugar import via the phloem.

The combined total of sugar eluted from, and remaining in, detached seed coats after perfusion was only 60% of the sugar content of intact seed coats (Table I). This may be partially explained by seed coat respiration, which would result in the loss of at least 7% of the sugar content of an intact seed coat over a 12-h period (estimated from data for attached-perfused soybean seed coats) (5). Also, perfusate was not collected during the initial 20 min of perfusion and this led to the loss of approximately 10% of the sugar content of intact seed coats (1.4 μmol of sucrose). The estimated seed coat respiration rate combined with the initial loss of sugar could not completely account for the low sugar content (eluted + retained) of detached-perfused seed coats. However, the respiration rate of detached-perfused seed coats may have been higher than estimated, and sugar may have been metabolized into unmeasured compounds during the perfusion period.

Tracer Efflux from Perfused Seed Coats

Tracer levels were insignificant at the beginning of seed coat perfusion (Fig. 2) because $^{14}CO_2$ assimilation commenced only 20 min prior to empty seed coat preparation. ^{14}C efflux from attached seed coats increased rapidly after 1 h of perfusion (Fig. 2). The rate peaked within a further 3 to 6 h and then declined slowly. During the initial 4 h of perfusion, tracer efflux was significantly increased by high osmoticum concentrations (Fig. 2, Table II). However, tracer efflux was not significantly affected by osmoticum concentration at any other time during perfusion, perhaps due to the high variability observed near the end of the perfusion period (Fig. 2). In addition, there was no statistically significant effect of osmoticum concentration on the total amount of tracer eluted during the full 12-h perfusion period (Table II).

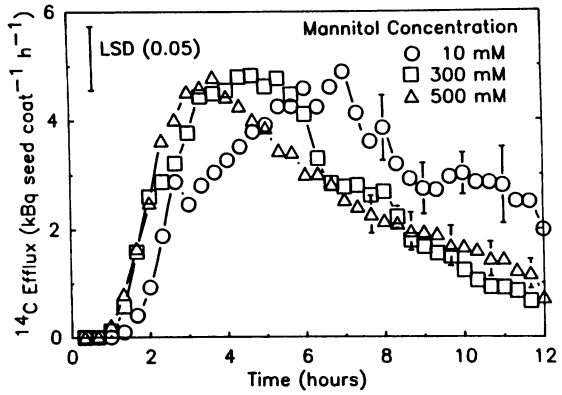

Figure 2. ^{14}C efflux from attached-perfused seed coats. Data for each mannitol concentration are the covariance adjusted means of three experiments (covariance was used to allow comparisons with Table II; significance of covariance: P = 0.24). The LSD (P = 0.05) between the 12 h mean efflux rates is shown. Standard error data are shown at hourly intervals for the final 5 h of perfusion. Tracer data were normalized as described in "Materials and Methods." Zero time refers to the beginning of seed coat perfusion.

High osmoticum concentrations significantly increased the ^{14}C content of the seed coat neutral and nonneutral fractions, and also increased the total ^{14}C content of the seed coat (Table II). The increase in seed coat ^{14}C content at high osmoticum concentrations appeared to follow the increase in seed coat sugar content (Tables I and II). The amount of ^{14}C in the seed coat nonextractable fraction was not affected by osmoticum

Table I. *Effects of Osmoticum on Sugar (Sucrose + Glucose) Elution from Seed Coats during 12 h of Perfusion*

Data is presented for the amount of sugar eluted, the amount remaining in the seed coat after perfusion, and the amount eluted plus the amount remaining in the seed coat for both attached and detached seed coats. Each data point is the mean of three replications; means with the same superscript in the same column are not significantly different at the 95% confidence level, except where indicated. Values in parentheses are the same data expressed as a percentage of the sugar content of intact nonperfused seed coats (the mean ± 95% confidence interval of intact seed coat sugar content was 14.70 ± 2.98 μmol [seed coat]$^{-1}$).

Concentration of Mannitol	Attached Seed Coats			Detached Seed Coats		
	Eluted	Seed coat	Total[a]	Eluted	Seed coat	Total
mM		μmol			μmol	
10	11.2a (76)	6.88b (47)	18.1b (123)	6.00a (41)	2.82a (19)	8.82a (60)
300	11.1a (76)	8.51b (58)	19.6ab (133)	4.97a (34)	3.48a (24)	8.45a (58)
500	11.2a (76)	13.1a (89)	24.3a (165)	5.37a (36)	4.07a (28)	9.44a (64)
$LSD_{0.05}$	4.08	3.82	6.16	2.25	2.79	3.18

[a] The significant differences of this column are protected at the 89% confidence level.

Table II. *Effects of Osmoticum on ^{14}C Elution from Seed Coats*

Data are presented for the amount of ^{14}C remaining in the seed coat after perfusion (12 h), the amount eluted during perfusion, and the amount eluted plus the amount remaining in the seed coat (also given as a percentage of the total ^{14}C in an intact seed: mean ± 95% confidence interval = 301 ± 19.2 kBq seed^{-1}). The average rate of ^{14}C efflux from the seed coat during the initial 14 h of perfusion is also presented. Data are the covariance-adjusted means of three replications (see "Materials and Methods"); the statistical significance of covariance (P value) is presented at the bottom of each column. Means with the same superscript in the same column are not significantly different at the 95% confidence level. Values in parentheses are the same data expressed as a percentage of the corresponding intact seed coat value (total intact seed coat ^{14}C content was 113 ± 7.81 kBq [seed coat]$^{-1}$); mean ± 95% confidence interval). Tracer data were normalized as described in "Materials and Methods."

Concentration of Mannitol	^{14}C Remaining in Seed Coat				^{14}C Eluted	Total Eluted + Seed Coat	Total as % of Intact Seed	Initial 4-h Efflux Rate
	Neutral	Nonneutral	Nonextractable	Total				
mM	*kBq*				*kBq*	*kBq*	%	*kBq h^{-1}*
10	24.8b (30)	13.3b (48)	4.49a (83)	43.2c (38)	32.7a (40)[a]	77.6a (68)[a]	25.8	1.60b
300	41.2a (50)	17.1a (62)	3.13a (58)	60.3b (54)	29.0a (36)	88.0a (76)	29.3	2.58a
500	51.1a (62)	17.1a (62)	4.42a (81)	73.2a (64)	29.1a (36)	102.0a (89)	33.9	2.63a
$LSD_{0.05}$	12.3	3.14	1.60	12.2	13.8	23.1		0.762
Covariance P value	0.04	0.003	0.05	0.016	0.23	0.044		0.24

[a] The percentage of intact seed coat data for this column was calculated using the intact seed coat neutral fraction.

concentration, so there was no evidence for osmoticum-induced changes in starch storage or mobilization (Table II). The increase in ^{14}C import to perfused seed coats at higher osmotic concentrations was not statistically significant (eluted + seed coat: Table II). However, this was probably due to the large variance and lack of osmoticum effects observed in the elution data (Table II).

The total amount of ^{14}C imported to perfused seed coats was approximately 30% of the amount imported to intact seeds (Table II). The use of a conservative seed size correction factor may have underestimated perfused seed ^{14}C measurements by as much as 27% (see "Materials and Methods"), but the large difference between intact and perfused seed ^{14}C import could not be fully accounted for by seed size underestimation. Thus, the lowered ^{14}C import by perfused seeds provides evidence for the inhibition of photosynthate import.

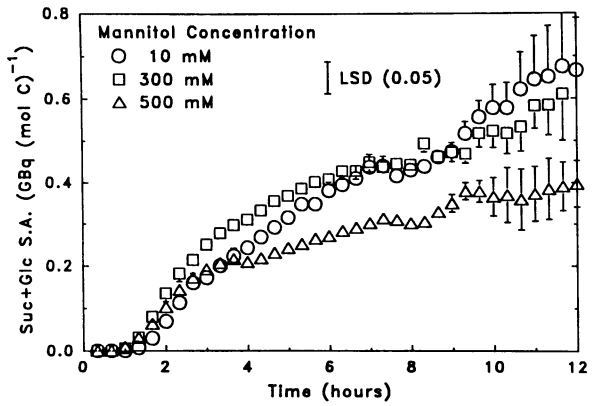

Figure 3. Specific activity of sugar (sucrose + glucose) in perfusate from attached seed coats. Data for each mannitol concentration are the mean of three experiments. The LSD (P = 0.05) between the 12 h mean sugar specific activities are shown. Standard error data are also presented. Specific activity data were normalized to a $^{14}CO_2$ specific activity of 1.0 GBq (mol C)$^{-1}$. Zero time refers to the beginning of seed coat perfusion.

Sugar-Specific Activity during Seed Coat Perfusion

One hour after beginning perfusion, the specific activity of eluted sugar was observed to increase continuously under all treatments (Fig. 3). The average of eluted sugar-specific activity over the full 12-h perfusion period was higher at lower osmoticum concentrations (concentrations below 500 mM; Table III). The rate of increase in eluted sugar specific activity between 2 and 10 h after beginning perfusion was also higher at lower osmoticum concentrations (Table III). Low osmoticum concentrations also appeared to increase the specific activity of sugar eluted during the final 3 h of perfusion (Fig. 3; Table III). However, this observation was not statistically significant, perhaps due to increasing variability during the final 3 h of perfusion (Fig. 3).

Perfusate sugar-specific activity measurements were overestimated by the analytical procedure and were thus not directly comparable with seed coat values (see "Materials and Methods"). However, the trend toward higher sugar-specific activities at lower osmoticum concentrations was also visible in data for sugar retained in the seed coat, despite the lack of statistical significance for this observation (Table III). Regardless of osmoticum concentration, the specific activity of sugar remaining in perfused seed coats was always lower than in intact seed coats (Table III).

Sucrose Uptake by Excised Seed Coats

The rate of sucrose uptake by excised seed coats after 1 h of elution (about 0.02 μmol sucrose [seed coat]$^{-1}$ h^{-1}; Fig. 4) was negligible in comparison with the rate of sugar efflux from detached-perfused seed coats after 1 h of perfusion (about 1.0 μmol sucrose [seed coat]$^{-1}$ h^{-1}; Fig. 1B). Osmoticum concentration had no significant effect on the rate of sucrose uptake in excised seed coats (Fig. 4).

Table III. *Effects of Osmoticum on Seed Coat and Perfusate Sugar (Sucrose + Glucose) Specific Activity*

Specific activity data from perfusates cannot be compared directly with seed coat data due to differences in analytical procedure (see "Materials and Methods"). Data is presented for the 12-h average of eluted sugar specific activity (S.A.) ("0–12 h"), the average over the last 3 h of perfusion ("Final 3 h"), and the slope of eluted sugar specific activity between 2 and 10 h after initiation of perfusion ("2–10 h S.A. slope"). Data are the means of three replications; means with the same superscript in the same column are not significantly different at the 95% confidence level. Values in parentheses are the same data expressed as a percentage of the intact (nonperfused) seed coat sugar specific activity (mean ± 95% confidence interval = 0.475 ± 0.106 GBq [mol C]$^{-1}$). Data were normalized to a $^{14}CO_2$ specific activity of 1.0 GBq (mol C)$^{-1}$.

Concentration of Mannitol	Seed Coat S.A.	Average Perfusate S.A.		Perfusate 2-10-h S.A. Slope
		0–12 h	Final 3 h	
mM	*GBq (mol C)$^{-1}$*	*GBq (mol C)$^{-1}$*		*GBq (mol C)$^{-1}$ h^{-1}*
10	0.386a (81)[a]	0.344a	0.621a	0.0576a
300	0.348a (73)	0.360a	0.549a	0.0411b
500	0.340a (72)	0.250b	0.377a	0.0287b
$LSD_{0.05}$	0.090	0.075	0.301	0.0138

[a] The means in this column were covariance adjusted ("Materials and Methods"; statistical significance: P = 0.013); other columns were not adjusted because covariance was not significant (P > 0.20).

DISCUSSION

Effects of Perfusion on Photosynthate Import and Efflux from Seed Coats

Import of ^{14}C to perfused seeds was approximately 30% of that to intact seeds (Table II). This observation differs from those made in pulse-labeling studies in which ^{14}C import to perfused and intact seeds was very similar (12, 18, 22, 24). The tracer fluxes produced by pulse-labeling are inherently unstable over time, and for this reason, pulse-labeled tracer accumulation in sink tissues (such as seeds) may not reflect the rate of photosynthate import over time (3, 17). The relatively stable export of tracer from steady-state-labeled leaves simplifies the analysis of tracer accumulation in seeds (2) (Fig. 2). Thus, the low import of tracer to perfused seeds observed in these experiments provides strong evidence that photosynthate import is reduced substantially by seed coat perfusion.

Photosynthate release from seed coats was also reduced by the perfusion treatment, as demonstrated by the lower proportion of total imported ^{14}C-photosynthate that was released from perfused seed coats (34%; average over treatments; Table II) compared to the proportion released from intact seed coats (62%, calculated from data in the legend to Table II). This result is consistent with the reduction in ^{14}C release from seed coats observed in surgically modified, unperfused seeds (2).

The inhibitory effects of seed coat perfusion on photosynthate import and efflux require that caution be used in interpreting the results of empty seed coat experiments (2, 10). However, the net fluxes of photosynthates observed in these experiments were well within an order of magnitude of the fluxes in intact seeds, and it is our opinion that the judicious interpretation of sugar and ^{14}C-photosynthate fluxes in perfused seed coats may provide insight into the mechanisms that regulate photosynthate unloading *in vivo*.

Physiological Components of Sugar Efflux from Perfused Seed Coats

Seed coats that were detached at the funiculus could not import sugar in the phloem. Thus, the higher sugar efflux of attached-perfused seed coats relative to detached-perfused seed coats (Table I) most likely resulted from the continued phloem import of sugar. For this reason, the difference in attached and detached seed coat sugar efflux (maximum of 1.0 μmol sucrose + glucose seed^{-1} h^{-1}, Fig. 1C) provides a measure of phloem import. This measure of phloem import was less than half of the rate estimated for intact seeds (2.0 μmol sucrose seed^{-1} h^{-1}) (2), and is thus consistent with the low ^{14}C-photosynthate import observed in perfused seeds. However, it remains unclear whether sugar efflux measurements provide an accurate measure of phloem import, because perfusing and/or detaching seed coats may alter seed coat efflux in unpredictable ways.

Sugar efflux measurements were the basis for Gifford and Thorne's (5) analysis of phloem unloading in soybean seed coats. The initial 2 to 3 h of sugar efflux from attached-perfused seed coats was attributed to a washout of sugar from the apoplast and symplast, whereas the "steady-state" sugar efflux after this period was said to equal the rate of continued phloem import and unloading (5). The identification of a "steady-state" efflux period by Gifford and Thorne (5) was based on the assumption that the rate of phloem import was constant throughout the perfusion period. In the current study, phloem import to attached-perfused seed coats was characterized by subtracting detached seed coat sugar efflux from attached seed coat sugar efflux (Fig. 1C). It is clear from the current analysis, that phloem import into attached-perfused seed coats is not constant over time (Fig. 1C). This result complicates the analysis of sugar efflux experiments.

Phloem import and/or unloading appeared to be inhibited at the beginning and end of the perfusion period, because there was no significant difference between attached and

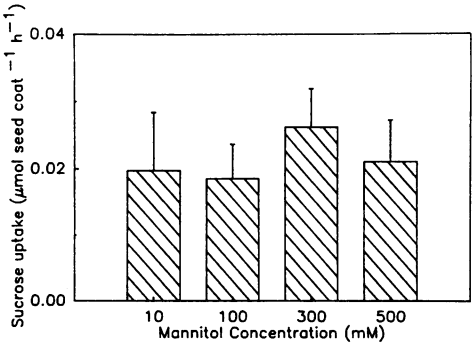

Figure 4. Sucrose uptake by detached seed coats. Uptake at each mannitol concentration represents the mean of five replications, and error bars are 95% confidence intervals.

detached seed coat sugar efflux at these times (Fig. 1C). The inhibition of phloem import and/or unloading near the end of the perfusion period is also apparent in data for ^{14}C-photosynthate efflux from attached-perfused seed coats (Fig. 2). Wolswinkel (19) and Gifford and Thorne (5) reported that sugar-efflux measurements were not useful for predicting phloem import and unloading until 2 h after beginning perfusion. These results are supported and extended by our observation that sugar efflux from seed coats was not enhanced by phloem import before 2 or after 8 h of perfusion. The reasons for the complex behavior of efflux are unclear. Therefore caution should be exercised in the interpretation of photosynthate efflux kinetics.

Properties of the Phloem Unloading Pathway in Perfused Seed Coats

The properties of sugar compartmentation in seed coats may reveal the pathway of phloem unloading. Most of the sugar in intact seed coats resides in tissues outside the phloem, because the concentration of sugar in the bulk symplast of seed coats is high (about 100 mM, see ref. 4), and the volume of phloem in seed coats is minute (11). The sugar content of attached seed coats perfused with 500 mM mannitol was similar to that of intact seed coats (Table I). This indicates that most of the sugar in attached-perfused seed coats was also in tissues outside the phloem (at least in the 500 mM treatment). Phloem import was required to replace the sugars lost from attached seed coats during the perfusion period, because the sugar content of attached-perfused seed coats was much higher than that of detached-perfused seed coats (500 mM treatment, Table I). The maintenance of a sugar content similar to that of intact seed coats by phloem replacement of sugars lost during perfusion thus demonstrates that sugars were transported to tissues outside the phloem in attached-perfused seed coats (500 mM treatment). Apoplastic transport from the phloem to other cells in perfused seed coats was probably retarded by the rapid washout of apoplastic solutes, so that symplastic transport was the only pathway for sugar movement within perfused seed coats. Thus, the similar sugar content of intact and attached-perfused seed coats (500 mM treatment) is best explained by the symplastic transport of sugars from the phloem to other tissues in the seed coat.

The case for symplastic phloem unloading is strengthened by measurements of seed coat sugar specific activity. Most of the sugar in seed coats was in tissues outside the phloem (see above). Thus, the specific activity of sugar in seed coats could not increase significantly unless phloem-imported tracer was transported to tissues outside the phloem. In intact seed coats, the transport of tracer from the phloem throughout the seed coat was demonstrated by the relatively high sugar specific activity reached in intact seed coats (relative to that of the path tissues) (2). In perfused seed coats, symplastic transport was the only pathway for tracer movement to seed coat tissues outside the phloem, because apoplastic transport was blocked by the loss of apoplastic solutes to the perfusion solution. Thus, a similar sugar specific activity in perfused and intact seed coats (assuming that sugar contents were approximately equal; 500 mM mannitol treatment, Table I) would clearly demonstrate a quantitatively significant amount of symplastic phloem unloading in perfused seed coats.

Indeed, the specific activity of perfused seed coat sugar was not much different from the intact control (Table III). The specific activity of sugar in perfused seed coats was as similar to that of intact seed coats (70% of the intact seed coat value, Table III) as was that of seed coats that were surgically modified but not perfused (66% of the intact seed coat value) (2). The specific activity of perfused seed coat sugar was very close to that of intact seeds considering that tracer import to perfused seeds was only 30% of the intact seed value (Table II). Therefore, the similarity of perfused and intact seed coat sugar specific activity demonstrates a quantitatively significant level of symplastic phloem unloading in perfused seed coats.

Some small degree of phloem unloading to the apoplast may occur in parallel with symplastic phloem unloading (20, 26). It is also possible that seed coat perfusion influenced the pathway of phloem unloading. However, symplastic phloem unloading is the most plausible explanation for the results of this study and suggests that most photosynthate is released to the seed coat apoplast across the greater membrane surface area of the many cells outside the phloem *in vivo* (11).

Turgor Effects on Photosynthate Import and Unloading in Perfused Seed Coats

The net import of sugar and ^{14}C to attached-perfused seed coats was enhanced by high osmoticum concentrations (Tables I and II). The primary effect of osmoticum is the modification of cell turgor in perfused seed coats (13, 14). Thus, the results of this study confirm that phloem import is enhanced by low cell turgor in perfused seed coats (1, 6, 9, 23, 25, 26).

High concentrations of osmoticum increased the initial (4 h) efflux of ^{14}C-photosynthate from perfused seed coats (Table II). However, no long-term effects of cell turgor on net sugar or ^{14}C-photosynthate efflux were observed in these experiments (Tables I and II). The lack of a substantial osmoticum effect on photosynthate efflux is in agreement with the investigations of Thorne and Rainbird (18) and Gifford and Thorne (5), but conflicts with the results of many other studies (1, 6, 9, 23, 25, 26).

When seed coat cell turgor was low, symplastic sugars were retained at a level similar to that in intact seed coats (Table I). On the other hand, high cell turgors caused a net reduction in seed coat sugar content (Table I). Thus, the partitioning of photosynthate between retention in the seed coat and release to the perfusion solution was sensitive to changes in cell turgor.

The turgor sensitivity of photosynthate release from perfused seed coats may be explained by the activity of a turgor-sensitive efflux control mechanism (6, 8, 26). Patrick (13) attributed the turgor sensitivity of photosynthate efflux to a turgor-sensitive sugar carrier that would increase photosynthate efflux when cell turgor was increased above a "turgor set point." In the current study, low cell turgor caused an increase in phloem import and an increase in photosynthate retention within the seed coat. This would lead to a buildup of symplastic solutes and an increase in cell turgor. Thus,

turgor-sensitive phloem import and seed coat unloading provide the basis for the "turgor homeostat" mechanism of photosynthate efflux regulation described by Patrick (13). Photosynthate efflux regulation by a turgor homeostat mechanism could provide a balance between photosynthate import and unloading *in vivo* (13).

Role of Apoplastic Retrieval in Photosynthate Unloading from Perfused Seed Coats

An alternate explanation for turgor-sensitive efflux is that there is a turgor-sensitive apoplastic retrieval mechanism in perfused seed coats (25, 26). Evidence for such a mechanism is based on efflux experiments with seed coats and embryos (25, 26). Efflux experiments cannot distinguish treatments that affect uptake from treatments that affect efflux. Therefore, efflux experiments are insufficient evidence for a turgor-sensitive uptake mechanism in seed coats. In our experiments, the rate of sucrose uptake by excised seed coats was negligible relative to the rate of sugar efflux in perfused seed coats (< 2%), and sucrose uptake was not affected by osmoticum concentration (Fig. 4). Thus, the results of the current study indicate that sugar efflux from perfused seed coats is not influenced by a turgor-sensitive apoplastic retrieval mechanism at the plasmalemma. The possibility of significant apoplastic sucrose retrieval in intact seed coats (turgor sensitive or otherwise) cannot be completely ruled out, because sucrose is highly concentrated in the apoplast *in vivo* (4, 15). However, all current evidence is against the existence of a turgor-sensitive apoplastic retrieval mechanism in seed coats, because previous results from perfused seed coats (25, 26) do not provide direct evidence for this mechanism. Thus, the best explanation for our observation of turgor-sensitive photosynthate retention within perfused seed coats is the turgor-sensitive efflux control mechanism described by Patrick (13).

Tracer Kinetics in Perfused Seed Coats

On first examination, the observation that increased phloem import (at higher osmoticum concentrations) was linked to a decrease in eluted sugar specific activity (Fig. 3, Table III) appeared to be anomalous. However, this result is consistent with symplastic phloem unloading and turgor-sensitive photosynthate retention within perfused seed coats. High osmoticum concentrations increased both phloem import and sugar retention within perfused seed coats (Tables I and II). Symplastic phloem unloading caused the mixing of phloem-imported tracer with the entire sugar pool of the seed coat before efflux to the perfusion solution. Therefore, increased phloem import did not lead to increased eluted sugar specific activity because increases in tracer import via the phloem were diluted by the larger seed coat sugar pool that resulted from retention of unlabeled sugar.

The trend of lower specific activities at higher osmoticum concentrations was also present in seed coat sugar specific activity data (Table III). However, the effect was not statistically significant, perhaps due to the increased variability of specific activity measurements near the end of the perfusion period (seed coat sugar specific activity was measured after perfusion; Table III; Fig. 3).

CONCLUSIONS

The empty seed coat technique significantly reduced both the import and efflux of photosynthates from perfused seed coats. However, physiologically significant levels of phloem import and unloading were measured in perfused seed coats, and a judicious interpretation of observations from empty seed coat experiments may provide information on photosynthate transport mechanisms *in vivo*. Taken together with the data from previous studies (1, 6, 9, 23–26), the current experiments provide compelling evidence that low cell turgor within perfused seed coats enhances net phloem import. The hypothesis that phloem unloading in seed coats is symplastic (11) was supported by measurements of tracer and sugar compartmentation in perfused seed coats. There was no evidence for a turgor-sensitive apoplastic retrieval mechanism in perfused seed coats. The partitioning of photosynthates between retention in the seed coat and unloading to the apoplast was turgor sensitive and could best be explained by the turgor homeostat model of photosynthate unloading described by Patrick *et al.* (14).

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