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Phloem Unloading in Soybean Seed Coats: Dynamics and Stability of Efflux into Attached 'Empty Ovules'

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ABSTRACT

The time-course of sucrose efflux from attached seedcoats (having their embryos surgically removed) into aqueous traps placed in the 'empty ovules' had three phases. The first phase lasted 10 minutes and probably was a period of apoplastic flushing. The second lasted 2 to 3 hours and is thought to be a phase of equilibration of seed coat symplast with the frequently refreshed liquid. The third phase of relatively steady efflux was postulated to reflect the continued import of sucrose from the plant, and hence to reflect the rate of sieve tube unloading. The average steady state efflux was equal under most conditions to the estimated rate of sucrose import. Efflux and import were unaffected by 150 millimolar osmoticum (mannitol or polyethylene glycol [molecular weight about 400]), by 0.5 millimolar $CaCl_2$, or by pretreatments up to 20 minutes with *p*-chloromercuribenzenesulfonic acid (PCMBS); they were enhanced by 40 micromolar abscisic acid, 40 micromolar indoleacetic acid, 20 micromolar fusicoccin, and 1 millimolar dithiothreitol (DTT) and were inhibited by 100 micromolar KCN, by 0.03% H_2O_2, by 20 micromolar and 5 micromolar trifluoromethoxy (carbonyl cyamide) phenylhydrazone, by repeated 5 minutes per hour treatments with 5 millimolar PCMBS, and by 5 millimolar DTT. The 'steady state' sucrose efflux was able to account for about half the rate of dry weight growth of the embryo, but stabilization of the system with <1 millimolar DTT taken together with other considerations is likely to give good correspondence between experimental unloading rates and *in vivo* growth rates.

Pulse-labeling experiments showed that ^{14}C-photoassimilate can pass from the parent plant into agar or liquid traps replacing the developing embryos in surgically prepared soybean seed coat 'cups' or 'empty ovules' that are attached to the plant (17, 22). Concurrently the seed coat cup tissues became heavily labeled. Thus the flow of solutes into an agar or liquid trap replacing the embryo is not necessarily identical to the flow unloaded from sieve tubes. Some molecules may be metabolized by the seed coat tissues (*e.g.* imported ureides converted to amides [12]) causing qualitative changes to the solute flow (16). Quantitatively the amount appearing in the trap may not equal the amount unloaded from sieve tubes reticulating the seed coat, until steady state has been reached. Legume seed coat tissue itself contains a sizeable pool of sucrose which can efflux into a liquid trap (24).

In the present study, efflux of total sucrose and amino acids into liquid traps, rather than of pulsed label, was the primary experimental focus. The objectives were: to follow in detail the time-course of efflux from attached seed coats in order to distinguish equilibration and steady state phases; to estimate the steady rate of sucrose import into surgically prepared empty ovules and to compare this rate with *in vivo* embryo growth rate; to test the effect on sucrose efflux and on import from the plant of various other solutes (*e.g.* metabolic inhibitors, osmotica) which have been shown to influence efflux of pulse-labeled solutes in this or related systems.

MATERIALS AND METHODS

Plant Material. A determinate soybean cultivar (*Glycine max* L. Merr cv 'Wye') was grown in a growth room as described previously (6) to provide a continual supply of uniform plants. For unloading experiments, plants were used 56 to 59 d after sowing (31–34 d after flowering), when the chosen pods contained embryos of 210 to 270 mg fresh weight and 26 to 27% dry weight.

Estimation of Seed Growth Rates. Rates of growth of individual identified seeds within intact pods were estimated by daily measurement of pod width at the widest point on the selected seeds (2). Pod thickness was determined, with an average standard error for each seed of 0.018 mm, from the mean of five measurements on each seed position using a micrometer gauge. For calibration the oven dry weight, w (mg), of 110 embryos (*i.e.* seeds less their seed coats) were regressed against the corresponding pod thickness, d (mm). A quadratic regression ($w = 44.8 - 23.4d + 3.91\ d^2$) fitted with $r^2 = 0.913$, over a range of thickness from 4 to 10 mm and plant age 56 to 65 d. The rate of growth of an individual embryo was approximated by so estimating the embryo dry weight at the same time of day on at least two successive days. Selected pods were used for unloading experiments immediately after the final thickness determination.

Preparation of 'Empty Ovules' or 'Seeds Coat Cups'. Experiments were conducted in the growth room in which the plants had grown, so there was no change in environmental conditions. The distal seed of three-seeded pods having pod thickness of 6.6 to 7.1 mm was selected. A pipe cleaner twisted around the stem was used to support the pod with its convex (dorsal) edge upward. Surgical preparation was similar to that used previously (17). Incisions were made so as to expose the distal (top) half of the distal seed through a window in the pod wall. The top half of the seed was cut off, and its seed coat separated from the half embryo and dropped in 1 ml 80% ethanol. A matching semicircular window was cut from the opposing pod wall so as to leave the dorsal vascular tissue intact as an arch. Silicone grease was brushed onto the cut edges of the attached seed coat cup to

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prevent or reduce wicking over the edge of liquid placed in the cup. The remaining half embryo slid out readily when a drop of water was eased into the cup/embryo interface by opening a slight gap between the half cotyledons. Use of appreciable force at this stage usually caused damage or breakage at the funiculus as evidenced by the failure of subsequent sucrose import from the plant (see below). The seed coat cup was filled with 65 μl of oxygenated water or aqueous solution. In most experiments the solution was changed every 20.0 min. If necessary the level of liquid was topped up with 5 μl water to replace evaporative loss although this was minimized by an Al foil hat placed loosely over the pod. The solutions collected were frozen until analysis. After each experiment the seed coat cups were snapped off at the funiculus and stored in 1 ml 80% ethanol.

Extractions and Analyses. Sucrose in 80% ethanol extracts of half seed coats was assayed with Worthington 'Flozyme' and invertase (6). The solutions taken from the cups were analyzed for glucose and sucrose using Flozyme and invertase, and occasionally for fructose using Flozyme and hexosephosphoisomerase. Amino acids were analyzed in lithium citrate on a Beckman model 119Cl amino acid analyzer. To measure cup area, the cups were cut down their sides to permit flattening and photocopying, prior to their oven-drying for weighing: the weight of the paper replica was used to calculate area.

Calculations. The loss of tissue sucrose from the seed coat cups during the course of the experiment was calculated as the difference between the sucrose content measured finally, S_{cf}, and the estimated initial sucrose content, S_{ci}. Gifford and Thorne (6) described how S_{ci} was determined via the sucrose content of the top half of each experimental seed coat.

The amount of sucrose imported through the funiculus, S_U (ng), during the course of an unloading experiment, was calculated as:

$$S_u = S_e - (S_{ci} - S_{cf}) + r_c \quad (1)$$

where S_e is the total sucrose appearance in the liquid trap during the entire experiment, r_c is the consumption of sucrose by respiration in the seed coat cup tissue during the whole experiment, and $(S_{ci} - S_{cf})$ is the decline in sucrose content of the seed coat cup tissue.

Seed coat cup respiration was measured separately on equivalent seed coats using a Clark-type O_2 electrode (Hansatech, King's Lynn, U. K.) at the temperature of the unloading experiments. Respiration of detached seed coat cups in buffer or water in the electrode cuvette showed only slight decline over 2 h and so it was assumed to be stable for attached importing seed coats. Respiration averaged 5.3 ± 0.2 μg (CH_2O) h^{-1} per mg of residual dry weight following alcohol extraction (n = 15) (alternative units; 0.91 ng $[CH_2O]$ [cm^2 cup area]$^{-1}$ s^{-1}). In the presence of 100 μM KCN, respiration was 55% of control, and in the presence of 20 μM FCCP[3], it was 145% of control. In the calculations (equation 1), it was assumed that imported sucrose either was, or replaced, the substrate of seed coat respiration. The calculation also assumes that there was no significant conversion of any seed coat starch to sucrose.

RESULTS

Time Course of Solute Efflux. Sucrose was the major solute appearing in the trap solution; three phases were distinguished in the time course of its efflux. In short experiments efflux declined very rapidly over the first 5 min but showed signs in some runs (Fig. 1) of stabilizing after 10 min. Glucose efflux showed a similar pattern at a lower absolute rate. Fructose efflux was trivial throughout. Amino acid efflux contributed 30 to 50% as much mass as did sugars.

The apparent plateau of efflux after 10 min when it occurred (Fig. 1) was short-lived and was never resolvable when samples were taken only every 20 min (Fig. 2a). Sucrose efflux continued to fall with time, though more gradually than over the first 5 min, typically reaching a minimum at between 2 and 3 h after the start, by which time glucose as well as fructose efflux was negligible. After that second phase, of gradually declining sucrose efflux, the most typical pattern in the third phase was for efflux to increase for 3 or 4 h after which it slowly declined again (Fig. 2, a and b). Sometimes the rise in rate after 2.5 h was minor or the curve simply plateaued at the minimal value. Seed coats that became detached at the funiculus during surgery exhibited a continuous decline in sucrose efflux to a virtual zero rate at 8 h (Fig. 2a, insert). It was evident that such seed coats were detached at the end of the run, because they offered less resistance to removal from the pod. They also had near-zero calculated import rates. Data from such seed coats were not used.

Amino acid efflux, determined just on a few seed coats, also exhibited these three phases except that there was only weak evidence of a 'hump' in phase 3 (Fig. 2b). Glutamine and asparagine were the dominant amino acids as noted previously (17). At the 2.5 h sucrose-minimum, amino acids contributed 31% as much dry weight as did sucrose to the efflux, while at the 4.5 to 5 h sucrose-maximum the amino acid contribution was 20%.

We interpret the first phase (~5 min) to be dominated by apoplastic purging of the seed coat (6). The significant contribution of glucose to efflux during that period and its disappearance later supports that interpretation since sieve tube sap does not normally contain appreciable hexose (*e.g.* 10). The second phase (5 min to 2.5 h) would be dominated by the equilibration of the symplastic sucrose in the seed coat tissues with the virtually sucrose-free liquid trap (*cf* 6). It was postulated that the third phase of sucrose efflux, starting at the 2.5 h minimum, might

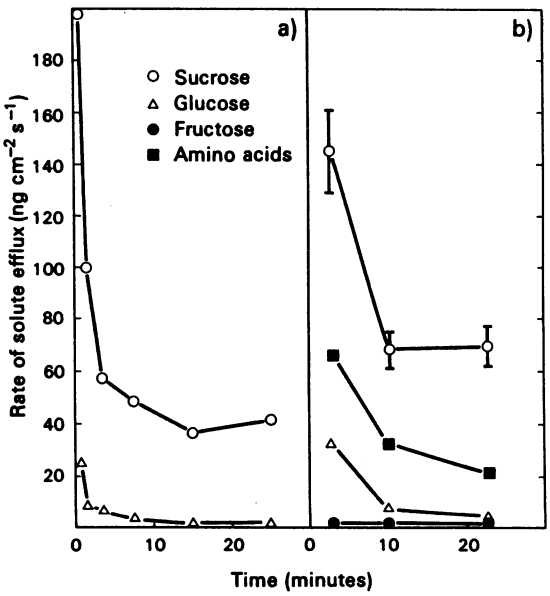

FIG. 1. Efflux of sucrose (○) glucose (△), fructose (●), and total amino acid (□) into oxygenated deionized H_2O over the first 25 min after placing water into attached seed coat cups. a, Mean of 4 replicates. b, Mean of 7 replicates for sucrose and glucose, with single-replicate subsamples for fructose and amino acids. Rates are expressed per unit area of seed coat cup surface.

[3] Abbreviations: FC, fusioccin; FCCP, trifluoromethoxy(carbonyl cyamide)phenylhydrazone; PCMBS, *p*-chlormercuribenzene sulfonic acid; PEG400, polyethylene glycol (mol wt ~ 400); PIPES buffer, piperazine-*N*,*N*'-bis(2-ethanesufonic acid).

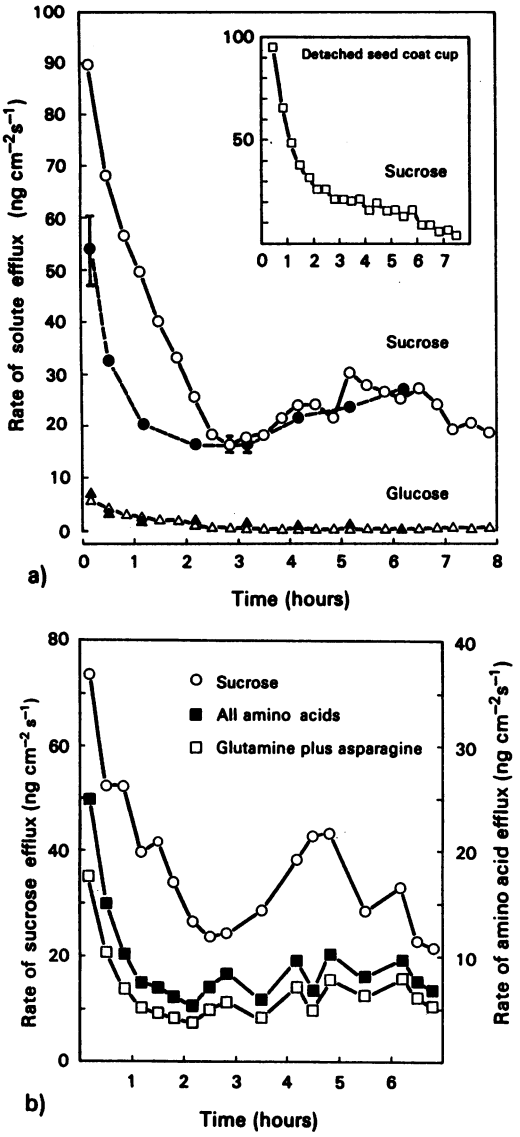

FIG. 2. Long term efflux of solutes from seed coat cups into aqueous traps. a, Efflux of sucrose and glucose in three experiments, two using attached seed coat cups (O——O, mean of 4 replicate seeds; ●——●, mean of 3 replicate seeds) and one involving a seed coat that became detached at the funiculus during surgery (inset). b, Efflux of amino acids in comparison with sucrose from a single seed coat.

reflect more closely the continued rate of import of sucrose from the mother plant through the funiculus. This was explored by comparing for attached seed coats the average rate of sucrose appearance in the liquid trap, after the minimum at 2.5 or 3 h, with the rate of import during the entire experiment as estimated using equation 1. These estimates of sucrose import and efflux were compared for 25 replicate seed coats effluxing into deionized H_2O or PIPES buffer, pH 6.3. There was no significant difference, the calculated rate of sucrose import over the entire experimental period being 21 ± 2 ng (cup s)$^{-1}$, and the average sucrose efflux, after the minimum had been reached, being 23 ± 2 ng (cup s)$^{-1}$. It is concluded that after the seed coat has equilibrated with a sucrose-free liquid medium for 2.5 h to 3 h, the rate of efflux of sucrose over the following 4 to 5 h is a measure of the rate at which sucrose is being imported into the seed and hence reflects the rate of phloem unloading at the time, and does not contain a component reflecting the partial washout of the seed coat tissue pool of sucrose.

Survey of Effects of Several Extracellular Solutes on Unloading. The above-described analysis of 'steady state' sucrose efflux (*i.e.* average rate of sucrose appearance in the liquid after 2.5 or 3 h equilibration) and of estimated sucrose import rate was repeated for experiments in which various solutions, buffered or unbuffered, were placed into attached seed coat cups (Table I). These experiments were to scan several possible modifiers to look for major effects. In all experiments the quantity of sucrose present in the seed coat cup tissue at the end of the experiment was less than it was estimated to be at the beginning of the experiment. The proportionate loss of cup tissue sucrose over the course of the experiment is also included in Table I. Since growing conditions, pod age and procedures were uniform, controls for each experiment were consistent and have been combined (Table I rows 1 and 2) into overall averages for water controls or 1 mM PIPES (pH 6.3) controls. Neither rate of import nor efflux were different in the presence of buffer than in its absence.

Generally there is excellent agreement in Table I between the average steady state efflux and the estimated import rate. The only exceptions were for H_2O_2 and for compounds that both penetrate cells and caused inhibitions (KCN, FCCP, and 5 mM DTT). This agreement supports the assumptions used in equation 1 to calculate import.

The presence of high osmotic concentration(150 mM mannitol or PEG400) had no effect on efflux, rate of import or the decline in seed coat sucrose content. Addition of $CaCl_2$ had no effect either. However, ABA, IAA, and FC all stimulated steady state efflux and import rate slightly, while the net loss of seed coat tissue sucrose content was not so great as for controls. None of these stimulations were notably large, the FC effect (50%) being the largest.

H_2O_2 (Table I, row 8) gave erratic results even though at 0.03% it caused no change in detached seed coat respiration rate as determined with an O_2 electrode. The reason for the discrepancy between average rate of import and efflux is obscure but, in any event, H_2O_2 did not stimulate unloading consistently. Prior oxygenation of the liquid seemed to suffice for aeration of the tissue.

KCN (100 μM) had no significant effect on the steady state sucrose efflux (Table I, row 9), while reducing the calculated rate of sucrose import by 60 to 70%. This can probably be explained by increased loss of seed coat tissue sucrose, which may have continued to lose sucrose during the apparently steady state period. That is, we suspect that with KCN present, steady state had not in fact been achieved between 3 h and 7 h from the start.

The respiratory uncoupler, FCCP, must have immediately stopped import for calculated import to average zero over the whole experiment. At the same time the seed coat tissue lost nearly all its sucrose and, while with 5 μM FCCP there was a semblance of steady efflux at a low level (2 ng cm^{-2} s^{-1}), in 20 μM FCCP efflux progressively decreased to virtually zero.

PCMBS applied as an initial pre-treatment of up to 20 min duration had no impact on efflux or import. However 5 min treatment each hour, or continuous treatment at one-tenth the concentration, increased the loss of sucrose from the seed coat tissue, while greatly reducing both import and steady-state efflux. Perplexingly, DTT at 5 mM (a reducing agent normally used to reverse the inhibitory effect of PCMBS pretreatment on membrane transport) was almost as effective an inhibitor of import and efflux as was continuous treatment with 0.5 mM PCMBS. However, 1 mM DTT increased efflux and import by about 50%, while reducing the decline of seed coat sucrose content.

Comparison of Efflux with Embryo Growth Rates. For 17 control seeds in 5 experiments the time-course of pod thickness for the seed studied was obtained for up to 3 d before the

Table I. *Effects of Various Solutes Placed into the Liquid Trap on Long Term (3–5 h) Steady State Efflux of Sucrose from Attached Seed Coat Cups following 2 to 3 h Equilibration*

Import rates, calculated according to the method in the text, used respiration rates determined separately in buffer or water as 5.3 μg (CH_2O) per mg extracted dry weight per h, or in the presence of KCN (2.9 μg mg^{-1} h^{-1}) or FCCP (7.7 μg mg^{-1} h^{-1}). PCMBS treatments were as pretreatments at the beginning of the equilibration period for times indicated, or as otherwise specified. NA means not applicable because efflux continued to decline throughout the experiment. Error limits are ± standard errors of the mean.

Row No.	Treatment	No. of Replicates	Buffered (Yes/No)	Calculated Import Rate	Steady State Efflux		Proportionate Loss of Cup Sucrose Content
				ng (cup·s)$^{-1}$	*ng (cup·s)*$^{-1}$	*ng cm*$^{-2}$ *s*$^{-1}$	
1	Water control	12	No	19.5 ± 1.6	21.7 ± 1.3	24.1 ± 1.4	0.55 ± 0.02
2	Buffer control	13	Yes	23.0 ± 2.1	23.6 ± 2.3	23.6 ± 2.1	0.49 ± 0.07
3	Mannitol, 150 mM	3	No	16.8 ± 4.7	15.5 ± 3.3	17.5 ± 3.2	0.54 ± 0.04
4	PEG400, 150 mM	3	No	22.7 ± 3.0	20.2 ± 2.6	22.6 ± 2.6	0.44 ± 0.06
5	$CaCl_2$, 0.5 mM	3	No	18.7 ± 3.0	19.2 ± 4.1	22.5 ± 4.7	0.45 ± 0.05
6	ABA, 40 μM	3	No	25.3 ± 0.9	23.9 ± 1.2	28.4 ± 1.1	0.30 ± 0.05
7	IAA, 40 μM	2	No	27.8 ± 4.9	26.9 ± 3.9	30.7 ± 4.9	0.38 ± 0.01
8	H_2O_2, 0.03%	3	No	12.6 ± 5.5	22.9 ± 4.8	27.2 ± 4.0	0.52 ± 0.11
9	FC, 20 μM	3	Yes	32.0 ± 2.4	35.4 ± 2.6	35.9 ± 3.6	0.41 ± 0.03
10	KCN, 100 μM	6	Yes	8.4 ± 2.4	21.7 ± 2.7	23.2 ± 3.2	0.63 ± 0.05
11	FCCP, 5 μM	4	Yes	−0.3 ± 1.7	2.2 ± 0.1	2.2 ± 0.2	0.88 ± 0.00
12	FCCP, 20 μM	3	Yes	−5.9 ± 2.5	NA	NA	0.93 ± 0.01
	PCMBS						
13	5 mM for 5 min	3	Yes	24.1 ± 5.2	26.0 ± 5.0	25.4 ± 4.3	0.49 ± 0.04
14	5 mM for 10 min	3	Yes	18.0 ± 2.7	18.2 ± 3.2	19.0 ± 3.1	0.49 ± 0.01
15	5 mM for 20 min	3	Yes	16.9 ± 5.1	21.7 ± 4.5	22.6 ± 4.1	0.51 ± 0.10
16	2 mM for 12 min	2	Yes	21.8 ± 8.3	21.3 ± 0.2	21.9 ± 1.2	0.53 ± 0.05
17	5 mM for 5 min each h	3	Yes	3.1 ± 1.3	6.3 ± 1.1	8.3 ± 1.6	0.68 ± 0.01
18	0.5 mM continuous	3	Yes	6.0 ± 4.3	5.9 ± 2.2	6.2 ± 2.0	0.68 ± 0.09
	DTT						
19	1 mM	3	Yes	31.1 ± 5.3	31.0 ± 1.6	31.2 ± 2.1	0.34 ± 0.09
20	5 mM	2	Yes	6.3 ± 1.5	13.4 ± 0.2	12.7 ± 0.6	0.67 ± 0.03

experiment. The dry weight growth rate of the seed was thereby estimated for each such seed over the 24 h preceding the experiment. It averaged 86 ± 8 ng s^{-1}. Since only half the seed coat was available for unloading, the relevant embryo growth rate for comparison is 43 ± 4 ng s^{-1}. Both the average steady state efflux and the average calculated import rate were 19 ± 1 ng (sucrose) s^{-1} for the 17 seeds. Thus the overall sucrose import rate was aproximately 50% of the half-seed growth rate.

DISCUSSION

In the absence of connection to the plant (Fig. 2a, inset) it took over 8 h of equilibration with a solute-free trap for the seed coat cells to cease losing sucrose at a steadily decreasing rate as also found by Wolswinkel and Ammerlaan (24) in pea. With sustained import from the plant through the funiculus, the decline in efflux of sucrose and amino acids into the liquid trap became arrested after about 2.5 h presumably because a dynamic balance was reached in the seed coat cells between import of sucrose from the plant and its disappearance from the seed coat (*i.e.* via efflux into the trap plus respiratory carbon loss). We consider that 'sieve tube unloading' should be equated most closely with import since the capacity of sieve tubes in the seed coat to act as a buffering pool is expected to be much less than the capacity of the rest of seed coat cells that occupy a much greater volume. Only at steady state can efflux into the trap be considered to reflect phloem unloading. The cells of the seed coat contain a large sucrose pool and readily take up or lose sucrose from the trap soution according to the sucrose concentration in the trap (6). While the processes involved in this exchange may well facilitate unloading, it is not, in itself, phloem unloading.

The partial recovery in sucrose efflux, peaking at about 5 h (Fig. 2), is perplexing. Total amino acids showed little evidence of such rallying (Fig. 2b), but it was more evident for certain components (notably histidine, threonine and asparagine; data not shown). Although such a time course was not found in other legume seed coat systems (21, 22, 25) there are data for attached pea seed coat cups (Fig. 3 in Wolswinkel and Ammerlaan [24]) for which a similar rallying was implied, though not commented upon.

The average rate of sucrose unloading from the soybean seed coat cups (about 20 ng s^{-1}) contributed about half that needed to explain measured embryo dry matter growth rate but the area for unloading must in fact be less than the measured cup area. There was a zone bordering the cut rim of the seed coat cup that was inactivated by damage. The grease brushed onto the cut edge moved into the tissue about 0.5 to 1 mm as evidenced by a translucent appearance. With the cup perimeter being 2.5 to 3 cm, this factor would decrease the area available for efflux by 0.13 to 0.3 cm^2 (*i.e.* by 13–30%). Also, the cup half of the seed coat includes the depression that houses the embryonic axis *in vivo*. This depression, occupying 0.08 to 0.1 cm^2, lacks the reticulated network of phloem (15) and therefore cannot have phloem unloading. The top half of the seed coat does not have such a phloem-free zone. These factors together with the 5 to 10 ng s^{-1} of amino acid unloading, increase the estimated efflux expressed as dry weight and adjusted for seed coat area available for sieve tube unloading, to a value close to the rate of embryo growth. However, this still does not account for any dry weight released by the embryo as respiratory CO_2; embryo respiration would be expected to add a further 25% to the import-need to sustain the embryo (8, 11). Thus the experimental system, using simple aqueous trap solutions, while not achieving rates of unloading fully commensurate with *in vivo* rates, can consistently

produce rates approaching *in vivo* rates. The shortfall might be due to dilution of essential apoplastic solutes, such as mineral ions, hormones, or osmotically active agents, owing to repeated change of trap solution. Some potentially important compounds were tested.

Various solutes had different effects on computed import rate, on steady state efflux into the aqueous trap, and on the loss of sucrose content of the seed coat tissue during the 6 to 8 h of the experiment. Adding the osmotica mannitol or PEG400 to the aqueous trap had no significant effect on import, efflux or on the reduction of sucrose content of the seed coat cup (Table I). This is surprising in that the sucrose concentration in the apoplast at the inner surface of the seed coat *in vivo* was 150 to 200 mM (6), and that the total osmolality in the apoplast would be much higher than that—maybe 350 mM (23). Nevertheless our result is consistent with the observation of Thorne and Rainbird (17) that mannitol up to about 500 mM—well above the plasmolyzing concentration—had no effect on sucrose efflux over 4 h. This result apparently stands in contradiction to that of Patrick (9), who found that sucrose efflux was decreased by osmotica such as mannitol in *Phaseolus vulgaris* seed coats. However, the experimental systems are not comparable: in the latter, short term efflux of label from detached seed coats, that had been prelabeled with ^{14}C-photosynthate before detachment, was temporarily reduced by including 100 mM osmoticum in the medium. The interpretation of results from such a non-steady-state system is difficult. Conversely, Wolswinkel and Ammerlaan (23) reported that high osmotic concentration enhanced the efflux of both radiolabeled sucrose and unlabeled sugar (23, 24) from attached *Vicia* and *Pisum* empty ovules.

Calcium ions at 0.5 mM, generally considered to be involved in membrane stabilization, had no significant impact on any of the parameters measured. Thus although addition of Ca^{2+} to the trapping medium is commonly routine (9, 17, 23), it may not be necessary. Using *Vicia faba*, Wolswinkel and Ammerlaan (22) also could not demonstrate an effect of Ca^{2+} compared with K^+ on efflux into solutions placed in attached empty ovules.

Both ABA and IAA at 40 μM caused a small increase in steady state efflux and import while reducing the loss of sucrose from the tissues of the cup (Table I). To the extent that the sucrose content of the seed coat represents a balance between input to it from phloem unloading and efflux from it into the aqueous trap plus seed coat respiration, this combination of effects is consistent with the hormones acting on the phloem unloading step (rather than on symplast to apoplast transfer from the seed coat parenchyma cells into trap solution) thereby reducing the 'draining' of these seed coat cells while enhancing net flow-through of sucrose from phloem to trap solution. This conclusion is, for ABA, consistent with the hypothesis that apoplastic ABA can enhance sieve tube unloading either by stimulating a proposed sucrose-proton cotransport mechanism (14) or by causing the phloem to be more leaky (20). Since actively growing soybean embryos contain high concentrations of ABA (13), the result suggests the hypothesis that developing embryos may be able to partially determine the rate of unloading by determining the concentration of apoplastic ABA. While Gifford and Evans (5) postulated that unloading might occur where sinks somehow cause phloem to be more leaky, the effectiveness of 20 μM FC (Table I) at enhancing import and efflux to the trap solution by about 50%, while reducing the decline of seed coat tissue sucrose, is more supportive of the idea of a degree of carrier-mediation involving proton/sucrose antiport. However, with respect to their effects on proton extrusion (ABA decreasing and FC increasing proton extrusion) the fact that both compounds increased import is difficult to interpret. As with ABA, FC inhibited the net loss of sucrose from the seed coat tissue, suggesting that the stimulation of efflux resulted from direct action on the phloem cells rather than on the seed coat closer to the trap solution. Using isolated half seed coats of fully expanded *P. vulgaris* seeds, Van Bel and Patrick (19) found that the efflux of preloaded ^{14}C-assimilate was *inhibited* over about 1 h by FC and stimulated by ABA. Again we presume that the apparent contrast with our results is related to the use of different techniques and the fact that different properties are being measured. However, the inhibition of ^{14}C loss from isolated seed coats by FC (19) is consistent with our inhibition of loss of seed coat sucrose by FC but neither process can be described as 'phloem unloading'.

The nonpermeant sulfhydryl reacting agent, PCMBS, is often used as a tissue pretreatment to distinguish carrier-mediated transport systems from passive ones (1, 3). It required either repeated 5 min booster treatments per h with 5 mM PCMBS or continuous treatment with 0.5 mM PCMBS to reduce sucrose efflux and import (Table I, rows 17–18). Single pretreatments up to 20 min were without effect (rows 13–16). Generally one expects continuous exposure to PCMBS to lead to its penetration of the plasmalemma whereupon it would be expected to inactivate virtually all enzymes. However, the agreement between calculated import rate and efflux in rows 17 and 18 (Table I) suggests that respiration rates (assumed to be equal to control rates in calculating import) had not been affected; hence PCMBS probably did not penetrate the cells. Those PCMBS treatments that reduced efflux and import from the plant, increased the drawdown of seed coat tissue sucrose. As for the ABA, IAA, and FC results, we take this to imply that PCMBS was acting principally on sieve tubes to reduce unloading and import, rather than on the surrounding seed coat cells. Surprisingly DTT—a compound expected, at 5 mM, to reverse the effects of PCMBS—was alone as inhibitory as 0.5 mM PCMBS. However, at 1 mM, DTT was almost as stimulatory as FC. This suggests that the seed coat cup system is sensitive to redox state and that even without PCMBS some sulfhydryl proteins important to unloading had become oxidized or altered. The DTT response, together with the need for continual or continuous exposure to PCMBS for its effect, suggests that PCMBS did not bind irreversibly to a specific sucrose carrier, but rather had a less specific effect relating to functional plasma membrane sulfhydryls. Apparently the PCMBS, even after 20 min reaction time, was washed away by subsequent changes of cup solution lacking the inhibitor.

Work with attached *Pisum sativum* and *Vicia faba* empty ovule systems (22, 25), indicated substantial inhibition of efflux of pulse-labeled solutes by continuous exposure to PCMBS. Whereas in Table I, PCMBS inhibition of import and efflux was accompanied by an enhanced loss of seed coat tissue sucrose, using pulse-label Wolswinkel and coworkers (25) found that PCMBS caused more label to accumulate in the seed coat tissue than in the control. Thus while with labeled substrate it was concluded that PCMBS acts by inhibiting release of substrate from the cells of the seed coat without affecting import (25), with nonlabeled sucrose efflux (Table I) we conclude that the PCMBS effect, where it occurred, was on sieve tube unloading (hence import) rather than on a transfer from the bulk of seed coat cells to the trap soution.

Elimination of phosphorylation by the uncoupler FCCP rapidly and completely stopped sucrose import, and allowed (or caused) the seed coat cells to almost completely lose their sucrose content. This suggests that sieve tube unloading is an energy-dependent process, while maintenance of the sucrose content of the ground tissue of the seed coat is dependent on continued input from sieve tube unloading. However, it cannot be ruled out that FCCP inhibited import by causing blockage of sieve-tubes.

The inhibition of import by cyanide was less than by FCCP. This may be because Cyt oxidase is not the only terminal oxidase available in respiration. In developing soybean embryos there is

a large cyanide resistant component to mitochondrial oxidation (18), and with our seed coats 100 μM KCN reduced O_2 exchange by only 45%. In fact, given that the alternative (cyanide resistant) pathway of respiration has a lower phosphorylation efficiency than the Cyt pathway (7), the degree of inhibition of import by KCN seems to be roughly commensurate with the degree of inhibition of respiration. The inhibition of import by KCN was probably by its acting on phloem unloading, rather than by blocking sieve-tube transport, because in *P. vulgaris* stems it took 10^3 × higher KCN concentration (100 mM) to block sieve tube transport (4). We interpret the failure of the steady state efflux to decline in the presence of KCN (Table I, row 10) as reflecting a delayed reaction of the extra loss of seed coat tissue sucrose in the presence of KCN—this delay of 3 h was noted by Wolswinkel *et al.* (25).

We conclude by highlighting methodological points. Sieve tube unloading (*i.e.* import), solute efflux into trap solution, and decline in seed coat sucrose content are all separate, though related, processes. Sharply different conclusions will be reached about control of imprecisely defined 'unloading', depending on whether unlabeled substrate or labeled substrate is investigated, whether attached or detached seed coats are used, and whether transitional or steady state phenomena are examined. To achieve absolute rates of assimilate import commensurate with rates of embryo gowth, inclusion of DTT at < 1 mM is advisable, but other inclusions tested such as Ca^{2+} did not seem necessary.

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