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Sugar Efflux from Maize (*Zea mays* L.) Pedicel Tissue[1]

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ABSTRACT

Sugar release from the pedicel tissue of maize (*Zea mays* L.) kernels was studied by removing the distal portion of the kernel and the lower endosperm, followed by replacement of the endosperm with an agar solute trap. Sugars were unloaded into the apoplast of the pedicel and accumulated in the agar trap while the ear remained attached to the maize plant. The kinetics of ^{14}C-assimilate movement into treated *versus* intact kernels were comparable. The rate of unloading declined with time, but sugar efflux from the pedicel continued for at least 6 hours and in most experiments the unloading rates approximated those necessary to support normal kernel growth rates. The unloading process was challenged with a variety of buffers, inhibitors, and solutes in order to characterize sugar unloading from this tissue.

Unloading was not affected by apoplastic pH or a variety of metabolic inhibitors. Although *p*-chloromercuribenzene sulfonic acid (PCMBS), a nonpenetrating sulfhydryl group reagent, did not affect sugar unloading, it effectively inhibited extracellular acid invertase. When the pedicel cups were pretreated with PCMBS, at least 60% of sugars unloaded from the pedicel could be identified as sucrose. Unloading was inhibited up to 70% by 10 millimolar $CaCl_2$. Unloading was stimulated by 15 millimolar ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid which partially reversed the inhibitory effects of Ca^{2+}. Based on these results, we suggest that passive efflux of sucrose occurs from the maize pedicel symplast followed by extracellular hydrolysis to hexoses.

Although assimilate partitioning in the sink tissues of plants is vital in determining the utilization of photosynthates, it is a process that is not well understood. Identification of the cellular and metabolic pathways involved in phloem unloading within plant sinks is vital toward future attempts at enhancing assimilate partitioning within crop plants (5, 17). Although the precise nature of phloem unloading is unknown, a common hypothesis is that the phloem tissues continuously reload sugars along the entire vascular length. In this view, the reloading process at active sinks is locally inhibited, thus permitting net unloading from the phloem tissue (9). The above concept is consistent with the concept of passive efflux from the phloem of storage sinks coupled with active uptake into storage cells (11, 23, 27). Ho and Baker (11) suggested that the above system, in which sucrose hydrolysis is often observed, is probably typical of storage sinks with apoplastic unloading, while symplastic unloading appears more likely in growth sinks such as leaves and root tips. The regulation of phloem unloading from the latter systems is postulated to be through the sink's growth rate, while in the storage sinks, regulation would be facilitated by changes in the permeability of the plasmalemma or by the apoplastic sucrose concentration (11).

In sugarcane, phloem unloading into the apoplast apparently occurs and is followed by hydrolysis of sucrose to hexoses prior to uptake by the storage parenchyma cells (10). A similar system may also be involved in maize kernels (3, 19–21). In sugar beet, however, sucrose storage within the taproot apparently occurs without hydrolysis (6). Precise determination of the unloading pathways in these species and in many other plant species is hampered by the lack of adequate techniques for studying the unloading process. Recently, Thorne and Rainbird (22) described a technique which they developed for mechanistic studies of assimilate unloading from soybean seed coats. Utilizing this technique, the above authors were able to study the unloading process through its responses to various chemical agents. Similar studies have been conducted on the seeds of other legumes (17, 25–27). In general, these studies have indicated that sugar and/or amino acid unloading from legume seed coats requires metabolic energy, since NaN_3, KCN, DNP[2], CCCP, NaF, PCMBS, and low temperatures inhibit unloading (17, 22, 26, 27). However, this type of system is probably not ubiquitous in crop plants since there is great diversity in the nature of the primary sink tissues.

This paper reports on an experimental system which was devised to study assimilate unloading from the maternal tissue of the maize kernel. The techniques employed provide a convenient method for studying sugar unloading without removing the ear from the plant and without removing the plant from the greenhouse or field.

MATERIALS AND METHODS

Plant Material. Plants of the dent maize (*Zea mays* L.) DeKalb hybrid, 'XL55', were grown in a greenhouse at University Park, PA. Ears from these plants were control sib pollinated and all studies were performed on ears aged 21 d postpollination. Ears of this age are in the active linear phase of dry weight accumulation and have usually attained less than 50% of their final dry weight. Greenhouse temperatures usually ranged from 20 to 32°C during the growth period. Agar efflux studies were conducted with intact plants in the greenhouse, so that normal conditions for assimilate transport prevailed during the course of the experiments. All experiments were started between 10:00 am and 12:00 noon local time. Natural lighting was utilized during clear days while metal halide lamps (Voight Lighting Industries, Philadelphia, PA) provided approximately 400 $\mu E\ m^{-2}\ s^{-1}$ at 1 m above the soil surface on cloudy days.

Scanning Electron Microscopy. Freehand longitudinal slices of basal tissue were cut from kernels before and after several of

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[2] Abbreviations: DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrozone; PCMBS, *p*-chloromercuribenzene sulfonic acid; MOPS, morpholinopropane sulfonic acid; MCW, methanol:chloroform:water (13:4:3, v/v/v).

the agar efflux experiments. Cytoplasmic contents of the cut surface cells were removed by digestion in 1% (w/v) bromelain (Sigma) in 0.1 M citrate-phosphate buffer (pH 4.5) for 72 h at room temperature (2). Several thymol crystals were added to inhibit microbial growth. The tissues were washed in several changes of deionized H_2O, dehydrated in an ethanol series, and dried in a critical point drying apparatus. The dried tissue was coated with 28 nm of gold using an ISI PS-2 coating unit and viewed with an ISI 60 SEM (International Scientific Instruments, Inc., Santa Clara, CA) scanning electron microscope at 10 kv.

Agar Efflux Treatments. Several rows of kernels were exposed by peeling back the husks on one side of an attached ear. The distal halves of several kernels were surgically removed with a scalpel. Experimental units consisted of groups of three kernels together on a row while replications usually consisted of similar groups on separate kernel rows. The remaining lower endosperms of the surgically altered kernels were gently pried from the kernel base with a microspoon spatula. Examinations utilizing both light and scanning electron microscopy indicated that the lower endosperm separated from the remaining kernel tissue just above the basal endosperm transfer cells. Therefore, for the studies reported here the remaining basal endosperm cells were carefully removed from the kernel base with a sharpened (No. 15) syringe needle in order to expose the tissues involved in assimilate unloading from the kernel base. After the above operations were performed, the remaining tissue of the kernel base formed a 'cup' consisting of the pericarp, vascular tissue, and pedicel parenchyma.

Experimental procedures varied slightly with individual experiments, but in general the altered kernels were immediately rinsed with deionized H_2O after removal of the basal endosperm transfer cells. Following this rinse, excess water was removed from the cup with a micropipette and the cup was filled for 10 min with 70 μl of a preincubation solution. The composition of the solution varied (inhibitors, water, buffers, $CaCl_2$, EGTA, etc.) depending on the objectives of the experiment. Following the preincubation period, excess solution was withdrawn from the cup and replaced with 70 μl of warm (38°C) 1% purified agar solution (Oxoid No. 3; Consolidated Laboratories, Inc., Chicago Heights, IL). The agar was prepared in 20 mM K-phosphate or MOPS buffer, pH 7, containing 0.1 mM PCMBS. It quickly solidified and formed a solute trap in contact with the pedicel cells. All PCMBS used in these experiments was purified in a column packed with Chelex 100 ion exchange resin (Bio-Rad Laboratories). Sugars, unloaded from the pedicel tissue, were allowed to accumulate in the agar matrix for 3 h. After 3 h the agar was removed from the cup and placed in 15 ml of methanol:chloroform:water (MCW, 13:4:3 v/v/v). In many experiments the pedicel cups were immediately refilled with agar or treated with a second preincubation solution. In this way a series of treatments and sugar collections could be carried out on one set of kernels. The agar-filled kernel cups were covered with parafilm and the remaining husks were wrapped around the ear during the collection period.

Sugar Analysis. All agar traps were stored in MCW at −20°C prior to extraction. For sugar extraction, the samples were allowed to warm at room temperature for 1 h and were then decanted into 300 ml evaporation vessels. The residual agar traps were then leached for 2 h with 15 ml of MCW followed by a final wash with 15 ml of MCW. All washes were combined and the methanol and chloroform removed by evaporation under reduced pressure at 40°C. After evaporation, each sample was brought to 25 ml with deionized H_2O, transferred to a plastic scintillation vial, frozen and held at −20°C for subsequent sugar analysis. Reducing sugars were determined by the alkaline ferricyanide procedure (15) modified for a Technicon Autoanalyzer II (Technician Instruments Corp., Tarrytown, NY) using glucose as a standard. Sucrose was calculated based on the amount of reducing sugar present before and after hydrolysis at 100°C for 15 min in 0.1 N H_2SO_4.

^{14}C Tracer Experiments. Plants were treated as described above so that agar-filled kernel bases were in place about 1 h prior to $^{14}CO_2$ pulse labeling. A portion of the ear leaf was enclosed in a plexiglass chamber (16 × 16 × 9 cm) and exposed to approximately 100 μCi $^{14}CO_2$ for 15 min. At various time intervals beginning at 90 min after labeling, agar traps and the associated kernel bases were removed separately from the ear, frozen, and freeze-dried. Adjacent intact kernels from the same ear were removed and dissected into the lower endosperm, kernel base, and distal kernel pieces. The freeze-dried tissues were pelletized in ashless paper and combusted on a Packard Tri-Carb model 306 sample oxidizer using Carbo-sorb II to trap the CO_2. The resulting ^{14}C samples were counted on a Beckman LS-8000 liquid scintillation counter.

RESULTS AND DISCUSSION

Anatomical Observations. Detailed study of assimilate unloading from the maternal tissue of the maize kernel necessitates that the endosperm be properly removed with a minimum of damage to the remaining tissues. Felker and Shannon (3) reported that the maternal tissue directly below the endosperm consists of the placento-chalazal tissue and the pedicel parenchyma. The cytoplasm of the pedicel parenchyma tissue is connected by numerous plasmodesmata. The vascular tissues divide and anastamose within the pedicel (3). Since no plasmodesmatal connection exists between the maternal tissue and endosperm, sugars must be unloaded into the apoplast prior to uptake by the highly invaginated basal endosperm transfer cells (3). At the plant growth stage used in these studies, the placento-chalazal cells are void of contents and possibly serve to increase the volume of the apoplast (3).

To study sugar unloading from the pedicel tissues into the apoplast, the basal endosperm should be neatly separated from the maternal tissues. Initial experiments and previous studies by Orr (14) indicated that the maize endosperm separates from the maternal tissue at the layer of thin-walled cells about three cell-layers above the basal endosperm transfer cells. Preliminary studies to establish a technique for the removal of the basal endosperm cells indicated that separation could best be achieved at approximately 21 d postpollination (data not presented). A sharpened (No. 15) syringe needle was used to carefully scoop the unwanted basal endosperm tissue from the pedicel.

Scanning electron micrographs (Fig. 1) of the pedicel cup after dissection indicated that the basal endosperm tissue was removed from the pedicel in most kernels. Removal of the lower endosperm using the above technique resulted in the formation of a 'cup' to which agar or other suitable trapping media may be added. The tissue forming the sides of the cup (Fig. 1) consisted primarily of spongy parenchyma cells which are not involved in the unloading process (3, 20). Tissues involved in assimilate transport within the kernel base (3) form the bottom of the cup (Fig. 1) and are in intimate contact with the agar trap. These tissues consist of the vascular tissues, pedicel parenchyma, placento-chalazal tissues (Fig. 2). Due to the nature of the tissue and the necessary dissection technique, it was not always possible to achieve ideal separation of the basal endosperm from the placento-chalazal cells. Since the placento-chalazal cells apparently are nonliving at the growth stage used for these experiments (3), the loss of these cells from the cup should not alter the validity of the present technique.

Thin sections were prepared from pedicels just after dissection and also after a 3-h collection period. Observation of these sections using light microscopy (data not shown) indicated that cell disruption by the dissection technique was confined to a

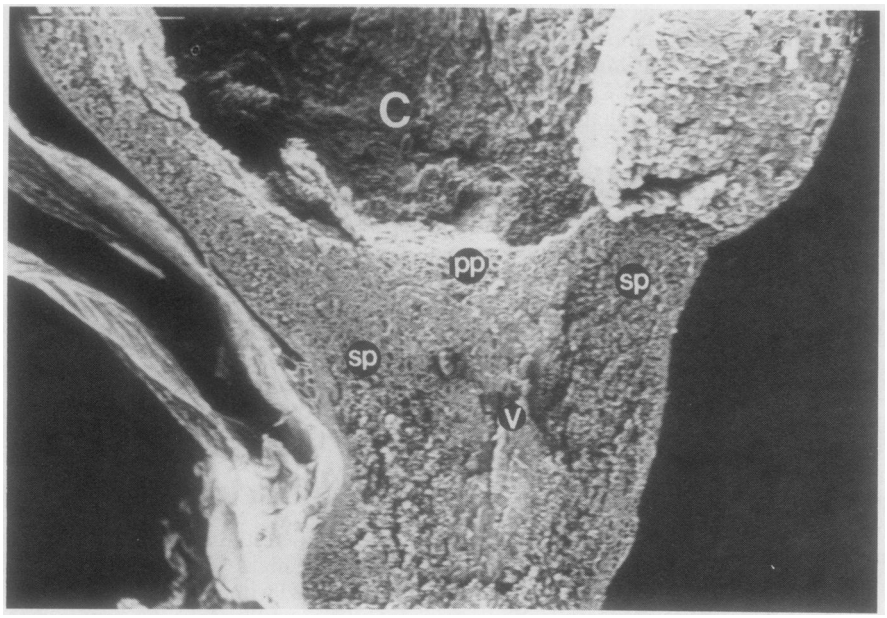

FIG. 1. Scanning electron micrograph of a longitudinal section through the base of a 21-d postpollination maize kernel. The endosperm and basal endosperm transfer cells have been removed. The resulting 'cup' can be treated with various solutions or inhibitors prior to application of an agar solute trap. V, Vascular bundle; sp, pedicel spongy parenchyma; pp, pedicel parenchyma; C, 'cup' (× 35).

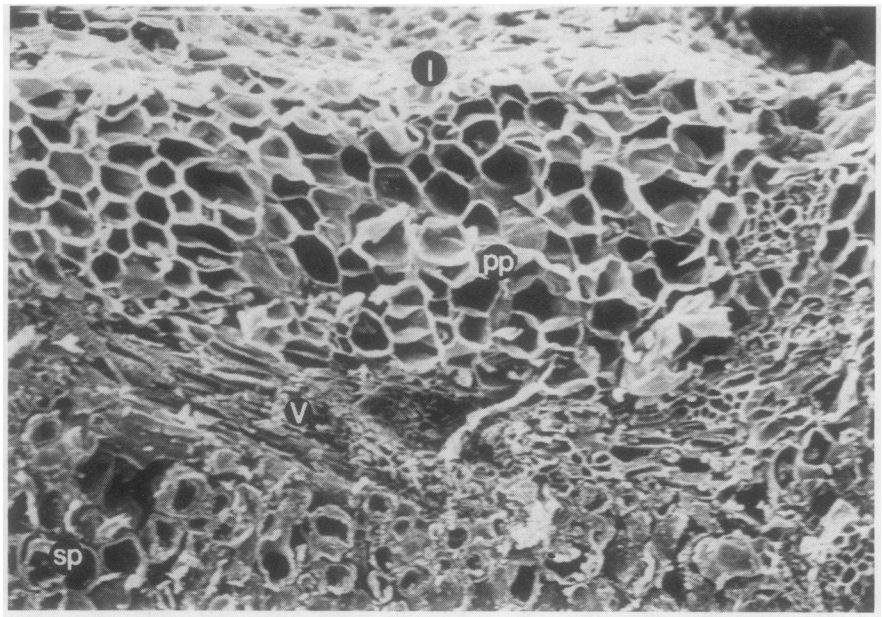

FIG. 2. Pedicel parenchyma cells and vascular tissue in the base of a 21-d postpollination maize kernel which has been prepared for introduction of an agar solute trap by removal of the basal endosperm transfer cells. Vascular bundles underlie several layers of intact pedicel parenchyma cells. V, Vascular tissue; sp, pedicel spongy parenchyma; pp, pedicel parenchyma; I, interface where basal endosperm cells and placento-chalazal cells were removed (× 200).

small fraction of cells near the surface and that cell contents were intact after the 3-h collection period.

^{14}C Tracer Studies. Tracer kinetics after $^{14}CO_2$ labeling were used to determine if the present technique closely simulated *in vivo* rates of assimilate unloading. Kinetics for the import of ^{14}C-photosynthate into agar solute traps (Fig. 3) were not significantly different from the kinetics of label movement into the lower endosperm of intact kernels. Within this time period essentially no ^{14}C moves into the upper endosperm. Both treatments produced similar upward curvilinear lines, suggesting that the present technique provides an accurate estimate of assimilate unloading from the pedicel tissue of intact kernels. Similarly, Thorne and Rainbird (22) observed that ^{14}C-assimilate movement into soybean seeds, altered with agar traps, was identical to that of intact seeds. Other studies on legumes have also suggested that assimilate unloading can continue for 8 h or more after the removal of the embryo and it was concluded that in legumes the embryo is not necessary for continued unloading from the seed coat (17, 22, 27). Similarly, in maize we found that the embryo and endsoperm are not necessary for the unloading of assimilates from the maternal tissue for at least 4 h.

Accumulation of ^{14}C-assimilate into the agar trap matrices and associated kernel bases was similar to that of intact kernels (Table I). Label moved into the altered kernels and was unloaded as if these kernels contained intact endosperm and embryo tissues. Removal of the distal kernel-half from otherwise intact kernels did not inhibit ^{14}C-assimilate movement into the remaining endosperm (Table I). During these short-term experiments, very little label moved from the lower endosperm into the distal portion of intact kernels (Table I). The presence of the basal endosperm cells in the pedicel cup restricted the movement of ^{14}C-assimilates into the agar traps, but had no effect on the amount of ^{14}C recovered in the kernel bases (Table I). Removal of the storage endosperm may disrupt the presumed active assimilate uptake and transfer by the basal endosperm transfer cells (3). Also presence of the basal endosperm cells would be an added barrier to the apoplastic diffusion of assimilates from the pedicel to the agar trap. Since we were interested in studying

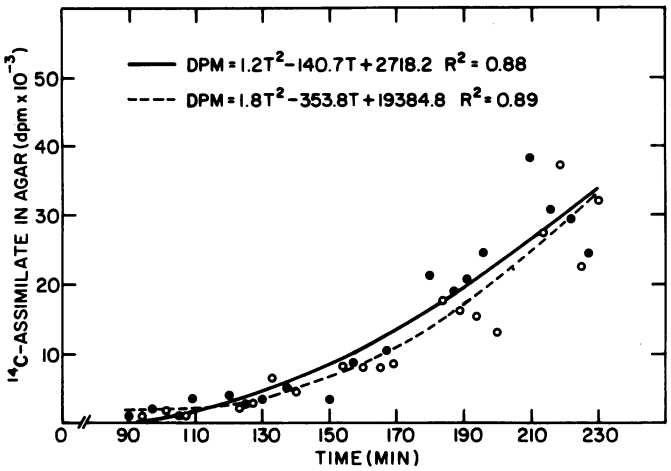

FIG. 3. Time course of ^{14}C-assimilate accumulation in agar trap matrices (●——●) compared with that of the lower endosperms of intact kernels (O- - -O). The ear leaf of a plant was exposed ($t = 0$) to 100 μCi $^{14}CO_2$ 1 h after imposition of the agar treatments. Each point represents one sample consisting of three kernels. Greenhouse temperature ranged from 19 to 21°C during this experiment.

unloading from the pedicel into the apoplast without the confounding effects of movement through or around the endosperm transfer cells, care was taken to be sure they were removed from the pedicel cups.

Effect of pH on Unloading. Active loading of sugars into leaf phloem involves proton co-transport and is sensitive to alterations of apoplastic pH (7). If assimilate unloading involves a reversal of this process, disruption of the existing proton gradients with buffers of varying pH would be expected to alter unloading rates. In maize, the free space of the pedicel contains acid invertase which presumably hydrolyzes unloaded sucrose prior to the uptake of monosaccharides by the basal endosperm transfer cells (19, 20). It has been suggested that invertase may hydrolyze sucrose during movement from the pedicel parenchyma cells into the apoplast or even within the cytoplasm of the pedicel parenchyma (14). If this is the case, invertase activity might regulate sugar unloading. Alternatively, free space hydrolysis may be a necessary prerequisite for the absorption of sugars into the endosperm via a monosaccharide specific active transport system (14).

In one experiment apoplastic pH within the pedicel was altered by utilizing agar traps with buffers of pH varying from 5 to 8, while in another experiment agar of varying pH was used in combination with 10-min pretreatment periods with the buffers. The sugar content of the agar traps was measured after a 3-h collection period. Sugar unloading was not influenced by the pH range used in this study (Table II). This indicates that sugar unloading from the maize pedicel probably does not involve proton cotransport. Similarly, Anderson (1) observed that the rate of sugar efflux from leaf discs was not affected by external pH, even though a dramatic effect on sugar uptake was noted. Extracellular pH also had no effect on sugar efflux from isolated mesophyll protoplasts (12). MOPS, Mes, and Tes, nonpermeant buffers, gave virtually the same results as citrate phosphate and K-phosphate buffers, which are considered to be permeant buffers (Table II). Several studies have demonstrated that sugar unloading from plant cells may be an ion-mediated process and that external potassium often influences the observed rate of unloading (1, 12, 22). Wolswinkel and Ammerlaan (27), however, observed that 25 mM K^+ had no consistent effect on phloem unloading in *Vicia faba*. The involvement of potassium cotransport in assimilate unloading within the maize pedicel is unlikely, since 20 mM K-phosphate gave virtually the same unloading rates in our study as did MOPS, which contained no K^+.

As the pH of the agar matrix was increased, an increase in the percentage sucrose collected within the agar traps was measured, suggesting an inhibition of the activity of acid invertase within the free spaces of the pedicel. This resulted in an increase in the percentage sucrose collected within the agar traps (Table II). The possibility that some inversion took place within the agar traps cannot be excluded in experiment III. In all later experiments, 0.1 mM PCMBS was incorporated in the agar to inhibit invertase activity within the agar traps. In experiment IV solutions of the same buffers and pH range described for experiment III were

Table I. *Effect of Several Kernel Dissection Treatments on ^{14}C Import by Maize Kernels*

The ear leaves of several plants were exposed to 100 μCi $^{14}CO_2$ approximately 1 h after dissection treatments were imposed. In the agar treatments, the endosperms were pried from their kernel base and in some treatments the basal endosperm transfer cells were removed. Agar was added to the resulting cup as a solute trap for studying assimilate transfer. Each value represents the mean of four replications ± SD, three kernels per sample. Greenhouse temperature ranges were 23 to 29°C for experiment I and 19 to 21°C for experiment II.

Kernel Component	^{14}C Content at Indicated Time (min) after Exposure			
	Exp. I		Exp. II	
	120	150	150	210
	dpm/3 kernels			
Agar-filled system with basal endosperm transfer cells removed				
Agar	3200 ± 1180	6500 ± 690	7700 ± 3800	30800 ± 5710
Kernel base	4100 ± 1330	7700 ± 1350	10400 ± 3150	22300 ± 4410
Agar-filled system with basal endosperm transfer cells present				
Agar			3800 ± 1440	14800 ± 2790
Kernel base			11700 ± 3070	21800 ± 3940
Half-kernel control				
Lower endosperm	4900 ± 1560	8300 ± 2790		
Kernel base	7100 ± 2720	10400 ± 1730		
Whole kernel control				
Distal kernel half	70 ± 40	80 ± 40	70 ± 40	600 ± 300
Lower endosperm	4000 ± 1640	9100 ± 2770	8300 ± 300	29800 ± 6200
Kernel base	5500 ± 2240	10400 ± 2800	14900 ± 2970	31900 ± 4630

Table II. *Effect of Varying the pH of the Agar Trap Matrix on Sugar Unloading in Maize Pedicel Cups and Accumulation in Agar Traps*

Dissection techniques were similar to those outlined in Table I for the treatment with basal endosperm removed. Following a brief water wash, excess water was replaced with 70 μl of 1% agar at the desired pH for experiment III. In experiment IV, the cups were washed briefly with water and treated with buffers at the desired pH. After 10 min, the residual buffer was removed and the pedicel cups were filled with 70 μl of 1% agar containing buffer at the desired pH and 0.1 mM PCMBS. Agar was removed after 3 h in both experiments. Percentage sucrose is based on moles of sugar. Values are means of four replications with three kernels per replication. Means followed by the same letter are not significantly different. Greenhouse temperature ranges were 17 to 20°C for experiment III and 26 to 29°C for experiment IV.

Treatment (20 mM Buffer)	Exp. III		Exp. IV	
	Total sugar	Sucrose	Total sugar	Sucrose
	mg/kernel·3 h	%	*mg/kernel·3 h*	%
Citrate-phosphate, pH 5	1.89	17.4 c	2.37	13.8 b
Mes, pH 6	1.75	22.4 b	2.44	15.6 ab
MOPS, pH 7	1.52	25.0 ab	2.50	22.4 a
K-phosphate, pH 7	1.69	24.6 ab	2.19	18.5 ab
Tes, pH 8	1.62	27.6 a	2.45	22.8 a
Waller-Duncan LSD K = 100	NS	3.6	NS	7.3

applied for a 10-min preincubation period followed by the addition of agar of the same pH which also contained 0.1 mM PCMBS. This procedure would be expected to more markedly influence the pH of the apoplast, yet the results were similar to those presented for experiment III (Table II). The insensitivity of unloading to variations in apoplastic pH and invertase activity, indicates that the unloading process is probably not controlled by apoplastic acid invertase activity.

Metabolic Inhibitors and PCMBS Studies. Sugar unloading from the seeds of soybean and broad bean, as well as unloading from other plant cells, is an energy requiring process and was shown to be markedly inhibited by a wide range of metabolic inhibitors (1, 17, 22, 26, 27). If sugar unloading from maize pedicel cells is also an active process, it should be sensitive to chemical inhibitors. In the present study, the application of various metabolic inhibitors and sulfhydryl group reagents was used to characterize sugar unloading from the maize pedicel. Sugar release from the pedicel was not affected by pretreatment with 2.5 mM PCMBS (Table III), a relatively nonpenetrating sulfhydryl group reagent and known inhibitor of phloem unloading in soybean seed coats (22) and loading in sugar beet leaves (8). In contrast, the percentage of unloaded sugars recovered as sucrose was significantly increased by PCMBS (Table III). PCMBS is a known inhibitor of invertase (14) and apparently the PCMBS pretreatment at least partially inhibited the apoplastic inversion of sucrose.

Sugar unloading from the maize pedicel was insensitive to 5 mM $HgCl_2$ (Table III), although this penetrating inhibitor also significantly inhibited hydrolysis of sucrose. CCCP, a penetrating respiratory uncoupler, had no effect on sugar unloading or the per cent sugar collected as sucrose (Table III). If the unloading process requires metabolic energy, a penetrating uncoupler would be expected to substantially reduce unloading over time. However, in this system CCCP had no effect on sugar unloading and accumulation in the agar traps for up to 5 h (Fig. 4). Similarly, pretreatment of the pedicel cups with the penetrating metabolic inhibitors, NaF and NaN_3, and DNP, a mitochondrial uncoupler, had no effect on sugar unloading and accumulation in agar (Table III). A second 3-h collection period following the above treatments indicated that NaF, NaN_3, and DNP had no observable effect on sugar unloading within 6 h of treatment (data not shown). The above treatments when applied in combination with 10 mM $CaCl_2$ gave results similar to $CaCl_2$ controls (data not shown).

Table III. *Effect of Several Chemical Agents on Sugar Unloading in Maize Pedicel Cups and Accumulation in Agar Traps*

Following a brief water wash, the appropriate aqueous solutions were added. After 10 min, the residual solution was removed, and water was added to the cup. Again after 10 min, the water was removed and the pedicel cups were filled with 70 μl of 1% agar containing 20 mM K-phosphate (pH 7) and 0.1 mM PCMBS; agar was removed after 3 h. Percentage sucrose is based on moles of sugar. In experiment VI, solutions in MOPS buffer were used as 10-min pretreatments, the excess was withdrawn from the cup, and the agar was added as above; agar contained 20 mM MOPS buffer (pH 7) and 0.1 mM PCMBS. Values are means of four replications with three kernels per replication. Means followed by the same letter are not significantly different. Greenhouse temperature ranges were 26 to 30°C for experiment V and 21 to 25°C for experiment VI.

Pedicel Cup Pretreatment	Total Sugar	Sucrose
	mg/kernel·3 h	%
Exp. V		
Water	1.61	12.3 c
PCMBS, 2.5 mM	1.58	26.4 b
CCCP, 0.2 mM	1.46	11.5 c
$HgCl_2$, 5.0 mM	2.00	35.1 a
Waller-Duncan LSD K = 100	NS	4.0
Exp. VI		
Buffer, 20 mM MOPS	1.13 ab	23.4
NaF, 5 mM	1.08 ab	22.5
NaN_3, 5 mM	0.99 b	23.8
DNP, 5 mM	1.20 a	26.4
Waller-Duncan LSD K = 100	0.15	NS

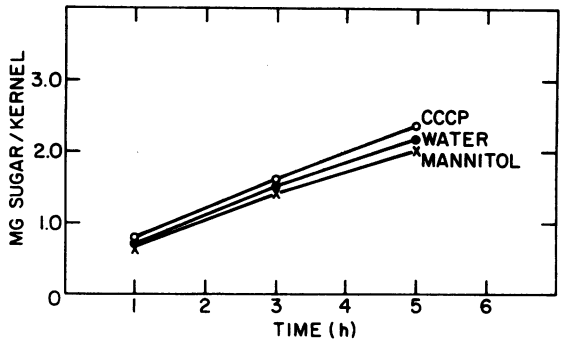

FIG. 4. Effect of CCCP and mannitol on release of sugars from maize pedicel cups and accumulation into agar traps. Agar matrices were 70 μl of 1% agar prepared in 20 mM K-phosphate (pH 7) containing 0.1 mM PCMBS. Agar matrices were removed and replaced by fresh agar at the designated times. One drop of water (●——●) or 0.2 mM CCCP (○——○) was added prior to each application of the agar traps. Mannitol, 200 mM (×——×), was included in the agar matrix of one treatment. Greenhouse temperature ranged from 26 to 30°C during this experiment.

The insensitivity of sugar unloading from the maize pedicel to penetrating and nonpenetrating inhibitors is in marked contrast to the systems described for soybean (22), pea (26), and broad bean (27). Thorne and Rainbird (22) observed a 75% decrease in ^{14}C unloading from soybean seed coats after treatment with 2.5 mM PCMBS, while 5 mM NaF and 1 mM DNP almost completely inhibited ^{14}C-assimilate transport into the treated seeds. Although our studies did not utilize ^{14}C tracers, studies by

Wolswinkel and Ammerlaan (27) demonstrated that PCMBS and low temperature substantially inhibited both sugar and ^{14}C-assimilate unloading from broad bean seed coats within 2 to 4 h of treatment. Wolswinkel and Ammerlaan (27) also demonstrated that NaN_3 and CCCP markedly inhibited ^{14}C-assimilate movement from broad bean seed coats. While sugar unloading from legume seed coats appears to be an active process, unloading from the maize pedicel appears to be passive.

The tissues between the terminal phloem elements and the embryo in soybean (24) are quite different from those between the phloem in the maize pedicel and the basal endosperm transfer cells (3). Based on an ultrastructural examination of the soybean seed coat tissues, Thorne (24) suggested that released assimilates may require passage through the symplasm of the endothelial layer. Such passage would likely require an active energy-requiring process, which could be perturbed by metabolic inhibitors. In maize the terminal phloem elements appear to be symplastically connected to the pedicel parenchyma cells, which are bordered by crushed pedicel cells and the dead placento-chalazal tissue, adjacent to the basal endosperm transfer cells (3). The basal endosperm cells of the maize kernel have a structure similar to transfer cells which might mediate sugar uptake into the endosperm (3). Perhaps the basal endosperm cells actively accumulate sugars from the apoplast of the pedicel tissue, while the sugars of the apoplast are replenished by passive diffusion from the pedicel parenchyma and vascular tissues.

In one experiment mannitol was added to the agar traps to determine if the osmotic potential of the matrix influenced sugar unloading. The amount of sugar accumulated in the agar traps containing 200 mM mannitol was the same as that of controls (Fig. 4). Exchange was nearly linear for both the control and mannitol treatments over a 5-h period. This is in agreement with the data of Thorne and Rainbird (22), which indicate that mannitol concentrations less than 500 mM have no effect on the amount of sugar unloaded from soybean seed coats. In contrast Wolswinkel and Ammerlaan (25) observed that ^{14}C-assimilate unloading from developing seeds of *Pisum sativum* was strongly inhibited by bathing solutions of both high and low osmolarity. An external osmolarity of 350 mM gave optimal rates of ^{14}C-assimilate unloading for pea (25). Patrick (16), however, has demonstrated that ^{14}C-assimilate unloading from excised seed coat halves of *Phaseolus vulgaris* L. was inhibited as the external solute concentration was increased from near zero to 100 mM. The observed differences in unloading responses to external solute concentration may be due to differences in treatment methods or to differences in response between these plants. These differences should be further investigated in future studies.

Since PCMBS is known to be a relatively nonpermeating sulfhydryl group inhibitor, several experiments were conducted to determine if PCMBS could be useful, as a preincubation treatment, for inhibition of extracellular invertase activity. In this way, the actual proportion of sugar unloaded from the maize pedicel cells as sucrose and the site of sucrose inversion within the pedicel could be more accurately determined. PCMBS, at concentrations ranging from 0.1 to 15.0 mM, was applied to the pedicel cup as a 10-min preincubation treatment. The excess PCMBS was washed from the pedicel cup with water. Used in this way, PCMBS did not significantly reduce sugar collected in the agar traps, but the percentage of sugar accumulating as sucrose increased as the PCMBS concentration increased (Table IV). When 5 to 15 mM PCMBS was used as the pretreatment, sucrose comprised about 60% of the sugar accumulating in the agar. Thus, it appears that at least 60% of the sugar unloaded into the free space of the maize pedicel is sucrose. The insensitivity of the unloading process to invertase inhibition indicates that extracellular invertase does not regulate the unloading process in this system. Whether sugar absorption by the basal endosperm transfer cells is regulated by extracellular sucrose inversion is not known at this time.

After the initial PCMBS pretreatment and 3-h collection period, 25 mM DTT was applied to the treated maize pedicel cups. DTT completely reversed the inhibitory effect of PCMBS on free space invertase so that there was no difference in the percentage of sucrose accumulating in the agar traps placed in any of the pedicel cups (Table IV). DTT did not inhibit sugar unloading (Table IV), but in this study the total amount of sugar accumulating was less during the second 3-h collection period. As discussed later, the reduction in sugar accumulation during the second 3-h period may be partly due to a plugging of severed sieve elements by callose.

Regulation of Sugar Release by Ca^{2+}. Calcium inhibits passive efflux of sugars from plant cells and its role in the maintenance of membrane structure and semipermeability has been demonstrated in several systems (1, 18, 22). Thorne and Rainbird (22) demonstrated that chelation of divalent cations, especially Ca^{2+}, from the plasmalemma by EGTA or EDTA caused rapid leakage of ^{14}C-assimilates from the seed coat. Calcium preincubation treatments were used in the present study to determine if Ca^{2+} influences passive efflux of sugars from the pedicel parenchyma and phloem tissue. Data presented in Table V indicate that 10 mM $CaCl_2$ significantly decreased the amount of sugars accumulating in the agar traps. The chelation of divalent ions, through the use of 15 mM EGTA, stimulated sugar efflux from the cells of the maize pedicel (Table V). These observations are consistent with the observations of Thorne and Rainbird (22) and Anderson (1).

A second pretreatment period and collection period were superimposed on the original agar treated kernels to determine if the effects of Ca^{2+} and EGTA are reversible. Following the original 10 mM $CaCl_2$ treatment with 15 mM EGTA resulted in a rapid stimulation of sugar efflux back to the water control level, but not to the level observed after double treatment with EGTA (Table V). Conversely, treatment of EGTA-stimulated kernels with 100 mM $CaCl_2$ decreased the sugar unloading and collection rate to that of the double Ca^{2+}–treated rate. Since the Ca^{2+} and EGTA effects are readily reversible, they are probably not due to alterations in cellular metabolism or to permanent deterioration of the plasmalemma. Because of the anatomy of the maize kernel, some damage to the pedicel is unavoidable during removal of the basal endosperm transfer cells. Thus, sugars accumulating in the agar traps likely move into the apoplast via both the plasmalemma of the pedicel parenchyma cells and the few sieve elements which were damaged. The decreased amount of sugars collected in the agar traps of water-treated pedicel cups during the second 3-h collection (Tables IV and V) is probably due in part to the plugging of damaged sieve elements. During the initial 3-h period, sugars would also move from the cut surfaces of other cells which were damaged during dissection. These sugars would contribute to the different amounts of sugar collected during the first and second 3-h collection periods (Tables IV and V). Figure 4 demonstrates that the damaged-cell sugars probably only contribute to sugar efflux during the 1st h of collection. Ca^{2+} reportedly stimulates callose formation in cut leaf petioles while EDTA tends to prevent blockage of sieve elements (13). Consequently, Ca^{2+} inhibits sugar efflux from cut petioles (13), while EDTA promotes continued sugar efflux (4, 13). EDTA apparently does not completely remove the callose plug once it forms (13). Other researchers have suggested that Ca^{2+} also reduces the permeability of the plasma membrane (1, 18, 22). In this study, we suggest that Ca^{2+} reduced sugar efflux due to both a reduction of the permeability of the plasmalemma and the stimulation of sieve element plugging. EGTA probably reversed the Ca^{2+} effect on the plasmalemma, since the second 3-h collection after EGTA treatment

Table IV. *Effect of PCMBS Concentration on Sugar Unloading in Maize Pedicel Cups and Accumulation in Agar Traps*

Procedures were similar to those outlined in Table III for experiment V. After the first 3-h collection, cups were treated for 10 min with 25 mM DTT (+) or water (−) and then washed 10 min prior to the second 3-h collection. Percentage sucrose is based on moles of sugar. Values are the means of four replications with three kernels per replication. Means followed by the same letter are not significantly different. Greenhouse temperature ranges were 23 to 25°C for experiment VII and 22 to 26°C for experiment VIII.

Pedicel Cup Pretreatment	1st Collection Period		25 mM DTT Treatment	2nd Collection Period	
	Total sugar	Sucrose		Total sugar	Sucrose
	mg/kernel· 3 h	%		*mg/kernel· 3 h*	%
Exp. VII					
H_2O	1.93	32.4 b	−	0.86	17.2 b
PCMBS, 0.1 mM	2.03	28.5 b	+	0.90	13.8 b
PCMBS, 0.5 mM	2.00	33.5 b	+	0.92	18.8 ab
PCMBS, 2.5 mM	1.93	55.8 a	+	0.85	26.3 a
PCMBS, 5.0 mM	1.92	60.6 a	+	0.75	19.3 ab
Waller-Duncan LSD K = 100	NS	9.8		NS	9.0
Exp. VIII					
H_2O	2.24	25.8 c	−	1.34	14.3 b
H_2O	2.00	20.4 c	+	1.17	12.1 b
PCMBS, 2.5 mM	2.12	40.0 b	+	1.33	19.2 ab
PCMBS, 7.5 mM	2.02	52.5 a	+	1.37	24.4 a
PCMBS, 10.0 mM	2.00	59.5 a	+	1.15	21.2 ab
PCMBS, 15.0 mM	1.85	57.3 a	+	1.17	18.6 ab
Waller-Duncan LSD K = 100	NS	8.4		NS	9.2

Table V. *Effect of $CaCl_2$ and EGTA on Sugar Unloading in Maize Pedicel Cups and Accumulation in Agar Traps*

Procedures were similar to those outlined in Table III for experiment VI; pretreatment solutions were in water. After the first 3-h collection, cups were treated for 10 min with appropriate solutions prior to a second 3-h collection. Percentage sucrose is based on moles of sugar. Values are the means of four replications with three kernels per replication. Means followed by the same letter are not significantly different. Greenhouse temperature ranged from 21 to 24°C during this experiment.

Pedicel Cup Pretreatment	1st Collection Period		Intermediate Pedicel Cup Treatment	2nd Collection Period	
	Total sugar	Sucrose		Total sugar	Sucrose
	mg/kernel· 3 h	%		*mg/kernel· 3 h*	%
Water	1.78 b	27.9 b	Water	1.24 b	24.3 b
$CaCl_2$, 10 mM	0.71 c	18.8 c	EGTA, 15 mM	1.16 b	16.6 c
$CaCl_2$, 10 mM	0.62 c	17.7 c	$CaCl_2$, 10 mM	0.38 c	15.3 c
EGTA, 15 mM	2.36 a	29.6 b	$CaCl_2$, 100 mM	0.42 c	17.7 c
EGTA, 15 mM	2.45 a	32.0 b	EGTA, 15 mM	2.20 a	33.5 a
$CaCl_2$ (10 mM) + PCMBS (2.5 mM)	0.54 c	40.2 a	$CaCl_2$ (10 mM) + DTT (25 mM)	0.41 c	18.7 bc
Waller-Duncan LSD K = 100	0.37	7.5		0.27	5.9

was equal to the water control and almost 3 times the Ca^{2+}-inhibited level (Table V). EGTA would not be expected to remove callose from damaged sieve elements (13), and thus, sugar efflux returns to the control rate, rather than the EGTA-stimulated rate (Table V).

In Table IV it was shown that PCMBS had no effect on the quantity of sugar accumulating in the agar traps. Since Ca^{2+} treatment reduced sugar efflux, apparently by stimulating the plugging of damaged sieve elements and by reducing the plasmalemma's permeability, we were interested in determining whether the remaining sugar efflux was sensitive to PCMBS. Again PCMBS had no significant effect on the amount of sugar collected in the agar trap (Table V). As noted earlier NaF, NaN_3, and DNP in combination with $CaCl_2$ had no effect on sugar accumulation in the agar traps. These data support our earlier conclusion that sugars move passively into the apoplast of the pedicel.

Sucrose comprised less than 20% of the sugar accumulating in the agar traps of Ca^{2+}–treated pedicel cups, but up to 30% following EGTA pretreatment (Table V). The agar in the pedicel cups contained 0.1 mM PCMBS, thus all sucrose inversion took place prior to its diffusion into the agar traps. Since PCMBS pretreatment allowed increased sucrose accumulation (Table IV), we concluded that much of the sugar moves into the apoplast as

sucrose. The higher sucrose percentage following EGTA pretreatment was probably due to a higher proportion of sucrose moving out of cut sieve elements and directly into agar traps before inversion could take place. Since sugars moving out of the pedicel parenchyma cells via the plasmalemma must diffuse through the pedicel apoplast and/or the placento-chalazal tissue prior to accumulation in the agar (3), this sucrose is more subject to hydrolysis by extracellular invertase. Consequently, the lower sucrose percentage accumulating after Ca^{2+} pretreatment probably resulted because a much higher proportion of sugars was unloaded through the plasmalemma into the apoplast. As noted above, the intermediate treatment with EGTA partially reversed the Ca^{2+} inhibition of sugar efflux, but it had no effect on the percentage sucrose accumulating in the agar trap. This is reasonable if we assume that EGTA is only increasing the permeability of the plasmalemma and that the unloaded sucrose is still subject to extracellular inversion prior to its accumulation in the agar traps. The conclusion that sucrose moves across the plasmalemma is supported by the results of the Ca^{2+} plus PCMBS pretreatment in which sucrose accounted for 40% of the sugars accumulating in the agar trap.

In conclusion, the described method provides a simple, valid technique for studying sugar unloading from the maternal tissue of the maize kernel. Using this technique, the unloading process may be challenged by various agents and important information with regard to the mechanism of sugar unloading can be obtained. The relative treatment effects are consistent between replications and treated plants. Some variation was observed, however, between experiments with respect to the amount of sugar unloaded and the per cent sucrose recovered. When adjusted for respiratory loss (approximately at 33%), the rates of sugar accumulation in the agar traps corresponded remarkably well with the 8.6 mg/kernel·d growth rate observed for this hybrid (14). Additional experimentation using this technique will be conducted to further characterize sugar unloading from the maize pedicel.

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