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Identification of a Membrane Protein Associated with Sucrose Transport Into Cells of Developing Soybean Cotyledons[1]

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ABSTRACT

The photolyzable sucrose derivative 6′-deoxy-6′-(4-azido-2-hydroxy)-benzamidosucrose (6′-HABS), competitively inhibited the influx of [^{14}C] sucrose into protoplasts from developing soybean (*Glycine max* L. Merr cv Wye) cotyledons. Photolysis of ^{125}I-labeled 6′-HABS in the presence of 10 millimolar dithiothreitol and microsomal preparations from developing soybean cotyledons led to label incorporation into a moderately abundant membrane protein with an apparent molecular mass of about 62 kilodalton (kD) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The 62 kD protein was partially protected from labeling by the inclusion of 100 millimolar sucrose in the photolysis medium and also by the inclusion of 10 millimolar phenyl α-D-thioglucopyranoside. Glucose, raffinose, or phenyl α-D-3-deoxy-3-fluoroglucopyranoside did not afford even partial protection from labeling. When the photolyzable moiety of 6′-HABS was attached to 6-deoxy-6-aminoglucose and ^{125}I labeled, the resulting photoprobe did not label the 62 kD protein above background. The labeled protein at 62 kD is therefore apparently a specific, sucrose binding protein. Sucrose influx into cotlyedons of less than 25 milligrams fresh weight (approximately 10 days after flowering) occurred by passive processes, but metabolically dependent uptake became dominant over the next 5 to 7 days of development. Both the Coomassie staining protein at 62 kD and label incorporation at that position in analysis of membrane proteins appeared concomitant with the onset of active sucrose influx. Polyclonal antibodies to the purified 62 kD protein bound specifically to a protein in the plasmalemma of thin sections prepared from cotyledons and density stained with colloidal gold-protein A. The results suggest that the 62 kD membrane protein is associated with sucrose transport and may be the plasmalemma sucrose transporter.

There is general agreement that a pressure mediated mechanism is responsible for the long distance movement of solutes in the phloem of higher plants. It is also generally agreed that this pressure is created as a consequence of the osmotic imbalance between elements of the transport tissue and the bulk of the leaf. The solute concentration step required to create this imbalance has not been unequivocally identified but, at least in plants that transport sucrose, seems to involve concentration from the apoplast (4). The transport involves a protein and the simultaneous import of protons (5, 7). In these aspects, phloem loading at the membrane level is analogous to the more completely characterized sugar/proton co-transport systems of bacteria and fungi (22). Neither the identity of the carrier protein nor its localization or concentration by cell type is known in leaves.

Study of a process which appears to be localized in a few cell types which are dispersed in a complex tissue is difficult both in interpretation of physiological experiments (21) and in obtaining abundant sources of the proteins involved. Sucrose influx in developing cotyledons of soybean appears to have many of the physiological characteristics of sucrose influx in leaf discs (16, 26) in that it requires a proton or electrochemical gradient for operation and apparently involves a carrier protein. We have used the sucrose transport system of developing soybean cotyledons (17, 24) in which sucrose transport probably occurs in all cells of the storage parenchyma (25) both as an interesting system in its own right, and as a possible model of transport into tissues of the phloem.

Structural requirements for substrate binding by the sucrose carrier in protoplasts derived from developing cotyledons (11) suggested that hydrophobic additions to the sucrose molecule could be tolerated, and in fact could be expected to enhance binding to the carrier if added at carbons 1, 3, or 6 of the fructose moiety. Success in utilizing aryl-azide derivatives of glucose to affinity label the glucose carrier in human erythrocytes (28), along with our findings led us to synthesize the photolyzable sucrose derivative 6′-deoxy-6′-(4-azido-2-hydroxy)-benzamidosucrose (6′-HABS),[2] whose structure is shown in Scheme I.

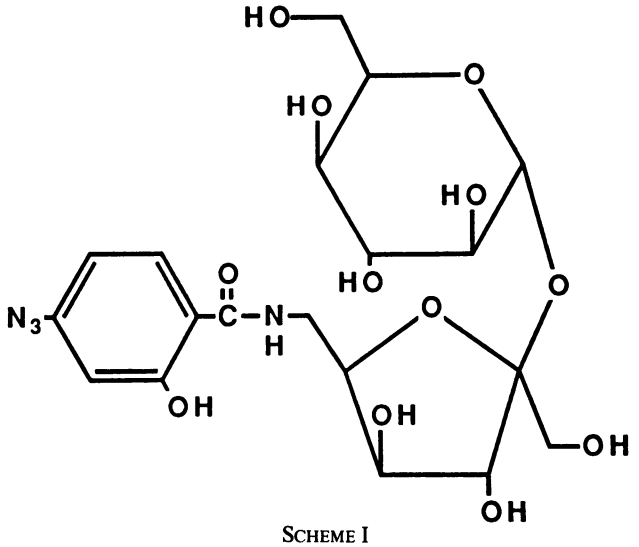
In the work presented here, we describe the synthesis, and use of 6′-HABS to identify a sucrose-binding, membrane protein in microsomal preparations from soybean cotyledons. Purification of the labeled protein allowed antibody production, and the use of those antibody preparations to begin molecular studies of the putative carrier are also described.

MATERIALS AND METHODS

Plant Material, Protoplast Preparation and Measurements of ^{14}C-Sucrose Influx. Soybeans (*Glycine max* Lin. cv Wye) were grown under controlled growth conditions and protoplasts from immature cotyledons were prepared as previously described (17). Inhibition of ^{14}C-sucrose influx into protoplasts by 6′-HABS was measured as described (11). Briefly, the influx reaction mixture contained approximately 5×10^5 protoplasts in 0.5 mL of media composed of 0.5 M sorbitol, 25 mM Mes-KOH buffer at pH 6.0, 0.5 mM $CaCl_2$, and varying concentrations of ^{14}C-sucrose and 6′-HABS. For analysis of inhibition by 6′-HABS at constant sucrose concentration, [^{14}C]sucrose was maintained at 0.2 mM while 6′-

[1] Contribution No. 4649.

[2] Abbreviations: AHB-GlcN, 6-*N*-(4-azido-2-hydroxybenzoyl)-D-glucpyranosylamine; 6′-HABS, 6′-deoxy-6′-(4-azido-2-hydroxy)-benzamidosucrose; OG, octyl β-D-glucopyranoside; PTG, phenyl α-D-thioglucopyranoside; 3-F-PTG, phenyl α-D-3-deoxy-3-fluoro-thioglucopyranoside; FCCP, *P*-(trifluoromethoxy)carbonyl cyanide; PTA, phosphotungstic acid.



SCHEME I

HABS concentration was varied from 0 to 200 μM. Inhibiton was also analyzed by varying [^{14}C]sucrose concentration from 0.2 to 5 mM and 6′-HABS concentration from 0 to 200 μM at each sucrose concentration.

Synthesis of Photolyzable Derivatives of Glucose and Sucrose. 6-N-(4-azido-2-hydroxybenzoyl)-D-glucopyranosylamine (AHB-GlcN) was synthesized as described by Harris *et al.* (9).

6′-HABS was synthesized starting from sucrose, and intermediates were characterized as described below.

2,3,4,3′,4′-Penta-O-acetylsucrose (**1**). Intermediate **1** was prepared as described by Buchanan, *et al.* (1) and had m.p. 151 to 158°C (lit. 154–156°C); $[\alpha]^{25}_D$ = +24.2°C, (c=1.02, $CHCl_3$). Calculated for $C_{22}H_{32}O_{16}$ C=47.83, H=5.84; found C=48.72, H=5.85.

2,3,,4,6,1′,3′,4′-Hepta-O-acetyl-6′-O-mesitylene-sucrose (**2**). The sucrose-penta-acetate **1** (6.16 g, 8.08 mmoles) in 80 ml of dry pyridine was stirred at room temperature under N_2 for 4 d with 8.08 mmol (1.756 g) of mesitylenesulfonyl chloride. Acetic anhydride (50 mL) was added and stirring was continued overnight. The reaction mixture was poured into 200 ml of water and extracted 3 times (300 mL) with CH_2Cl_2; the CH_2Cl_2 layer was washed 3 times with 1 N HCl (300 ml) and twice with water (200 mL) and was dried over sodium sulfate. Flash chromatography on silica eluted with 4:1 ether:hexane (v/v) gave pure **2** as an oil (2.47 g, 37.6%). $[\alpha]^{25}_D$ = +47.4 ± 0.8°C. Calculated for $C_{35}H_{46}O_{20}S$, C=51.34, H=5.66; found C=51.15, H=5.5. The 360 mHz ^{1}H-NMR spectrum in $CDCl_3$ showed the presences of seven acetates and the mesitylene group.

2,3,4,6,1′,3′,4′-Hepta-O-acetyl-6′-deoxy-6′-azidosucrose (**3**). The mesitylene sucrose **2** (2.47 g, 3.02 mmol) was held at 80°C with 0.196 g (30 mmol) of sodium azide in 25 mL of DMF for 18 h under N_2. TLC analysis (silica, 4:1 ether:hexane, v:v) indicated about 80% conversion to a component of higher mobility than the starting **2**. The cooled reaction mix was added to 50 ml of water and extracted with CH_2Cl_2 (3 times, 150 mL). The CH_2Cl_2 layer was washed with water (2 times, 100 mL) and reduced to a syrup under high vacuum. The syrup was redissolved in ether and passed through silica (70 mL) to remove a slight discoloration. Reduction to a glass which crystallized upon prolonged standing gave **3** (1.56 g, 2.36 mmoles, 78%) which had m.p. 136-141°C, $[\alpha]^{25}_D$ = +55.4 ± 0.8°C (c=0.98, $CHCl_3$). Calculated for $C_{25}H_{35}O_{17}N_3$, C=47.20, H=5.33, N=6.35; found C=47.44, H=5.30, N=6.46. The IR spectrum in $CHCl_3$ showed a prominent absorbance at 2100 cm^{-1} (N_3 stretch). To confirm the position of the azido group, **3** was further characterized by ^{13}C-NMR in D_2O after deacetylation of a small sample. All signals were singlets; their chemical shift in ppm from tetramethylsilane and assignment in the molecule are as follows: (primed numbers refer to the fructose moiety) 52.7, C-6′(shifted from 60.0 in sucrose); 60.2, C-1′; 60.9, C-6; 69.2, C-4; 70.8, C-2; 72.3, two carbons unresolved, C-3 and C-5; 75.2, C-4′; 76.0, C-3′; 79.47, C-5; 92.0, C-1; 103.67, C-1′.

2,3,4,6,1′,3′,4′-Hepta-O-acetyl-6′-deoxy-6′-(4-azido-2-hydroxy)-benzamidosucrose (**5**). The azide **3** (400 mg, 0.605 mmol) was dissolved in 7 mL of ethanol, 1 equivalent of HCl was added and the solution was cooled to 0°C, placed under an N_2 atmosphere, and about 50 mg of 10% Pd on C was added. Hydrogenation was carried out at 0°C and 15 p.s.i. H_2 for 4 h. TLC (silica, 9:1 ethyl acetate:methanol, v/v) showed conversion to a single, ninhydrin positive component. 2,3,4,6,1′,3′,4′-Hepta-*O*-acetyl-6′-deoxy-6′-aminosucrose (**4**) was not stable in neutral solution due to self deacetylation. The acidified, ethanol solution of **4** was filtered through Celite, reduced to a syrup, and used without further characterization. The syrupy **4** was dissolved in 10 mL of CH_2Cl_2, 0.75 mmoles of *N*-hydroxysuccinimido-4-azidosalicylic acid (13) was added along with 5 mL of water. The aqueous phase was made just basic with triethylamine and the reaction mixture was stirred at room temperature for 10 h. The CH_2Cl_2 layer was separated and washed twice with water (20 mL). Intermediate **5** was separated from a contaminant of greater mobility by flash chromatography on silica eluted with 9:1 ether:hexane (v/v) to yield 240 mg (0.30 mmol, 49.8% from **3**) as an amorphous solid. Calculated for $C_{33}H_{40}O_{19}N_4$, C=49.75, H=5.96, N=7.03; found C=59.59, H=5.09, N=7.23. The 360 mHz ^{1}H-NMR in $CDCL_3$ was consistent with the expected structure.

6′-Deoxy-6′-(4-azido-2-hydroxy)-benzamidosucrose (**6**). Solid **5** (180 mg, 0.226 mmol) was dissolved in 5 mL of 1:1 ether:methanol (v/v), a small amount of sodium metal was added, and the mixture was stirred at room temperature for 2 h. After 2 h an additional 5 mL of methanol was added to dissolved a white precipitate which had formed and the reaction was allowed to continue for 1 h. TLC analysis (silica, developed with 7:3 ethyl acetate:methanol, v/v) showed conversion to one product of low mobility. The solvents were removed under vacuum and the solid was dissolved in 1:1 methanol water (v/v). A slight yellow color was removed by extraction with ether (3 times, 3 mL) and the product was passed through a 1 mL column of Dowex-50 (OH^- form) to deionize. Removal of solvents under vacuum followed by drying by repeated evaporation of ethanol gave **6** as an amorphous solid (103 mg, 0.206 mmol, 91%). Calculated for $C_{19}H_{26}O_{12}N_4$, C=45.33, H=5.41, N=11.13; found C=44.92, H=5.52, N=10.93.

^{125}I-Labeling of 6′-HABS and AHB-GlcN. Twenty μl of 0.1 M potassium phosphate buffer (pH 7.2) containing 5% glucose and 1.4 nmol of 6′-HABS or AHB-GlcN was added to 1 mCi of Na^{125}I in 10 μL of solution at pH 10. The reaction was started by the addition of Enzymobeads (Bio-Rad) in 25 μL of the same buffer. The reaction was allowed to proceed for 1.5 h and most of the water was removed under an N_2 stream which was passed through an activated carbon filter at the exit of the reaction vessel. The residue was redissolved in 1:1 methanol:water (v/v), applied to a 4 cm origin on a plastic backed, silica TLC plate (Polygram Sil G, Macherey-Nagel, FRG), and developed with 7:3 ethyl acetate:methanol (v/v). ^{125}I-products were localized by a short exposure autoradiograph produced by placing the developed TLC plate on foil-wrapped film and by correspondence of the product band to the product on a corresponding TLC developed in the same chamber and visualized by charring. The product band was cut from the plate and eluted by placing the plate zone in 10 mL of methanol. Total yield was 412 μCi to give a minimum specific activity of 292 Ci mmol^{-1} assuming complete recovery of the starting sugar derivative. The labeled

probe was stored as the dilute solution in metanol, dried and redissolved in the appropriate buffer just prior to use.

Photolysis of Microsomal Preparations from Developing Soybean Cotyledons with ^{125}I-6′-HABS or ^{125}I-AHB-GlcN. Pods were removed from plants at 19 to 21 DAF and cotyledons were removed from the pod and seed coat. Cotyledons were ground at 4°C in a food processor using a buffer consisting of 250 mM sucrose, 2.5 mM DTT, 10 mM EGTA, 10 mM $MgSO_4$, and 0.5% gelatin (w/v) in 25 mM Mes-KOH at pH 7.0 at 1.5 mL buffer per gram of cotyledons. The ground mixture was squeezed through three layers of cheesecloth and centrifuged twice at 13,000*g* for 10 min each time. The supernatant was then centrifuged at 85,000*g* for 30 min, and the microsomal pellet obtained was washed three times by resuspension in 10 mM DTT, 10 mM Mes-KOH at pH 6.0 followed by centrifugation at 85,000*g*. The washed microsomal preparation was resuspended in the DTT/Mes buffer at 30 mg protein/ml and stored in liquid nitrogen until used.

The photolysis mixtures consisted of 0.75 mg of microsomal protein, 5 μCi of either ^{125}I-6′-HABS or ^{125}I-AHB-GlcN (to give a photoprobe concentration of 0.57 μM), 10 mM DTT and the desired concentration of protecting sugar in some cases, all in 30 μl of 10 mM Mes-KOH at pH 6.0. The mixtures were prepared at 0°C and either photolyzed as droplets on a plastic surface at 0°C, or quickly frozen onto a glass cover slip which was placed on a brass rod which was in turn immersed in liquid nitrogen. In either case the droplet was photolyzed using a UV lamp with maximal output at 365 nm (Mineralite, 400 μW/cm^2). Frozen droplets were transferred to a −20°C freezer, maintained frozen overnight, thawed quickly and washed as described below.

Photolyzed droplets were diluted with 1 ml of 10 mM Mes-KOH, pH 6.0 buffer, centrifuged at 13,000*g* for 20 min, the supernatant was discarded and the pellet was washed twice more in the same way. Protein was precipitated from the final pellet by addition of 1 mL of 8:2 (v/v) CH_2Cl_2:methanol and pelleted by centrifugation at 20,000*g* for 10 min. The pellet was resuspended in the same solvent, again centrifuged, resuspended in 1 mL of ether, centrifuged and dried under a nitrogen stream.

Proteins were solubilized in 120 μL of solubilization buffer consisting of 2% (w/v) lithium dodecylsulfate, 50 mM Tris, 50 mM DTT, and 7% (v/v) glycerol at pH 6.8 by heating at 37°C for 40 min.

SDS-PAGE was carried out using the Laemmli buffer system (15), and a 0.75 mm, 9% resolving gel run at 55 mamp for 1 h at 14°C. Gels were stained with 0.3% Coomassie blue R-250, destained in 5% methanol, 7% acetic acid and dried on cellophane. Dried gels were autoradiographed at −80°C on Kodak X-Omat AR Film in cassettes equipped with Cronex intensifying screens (DuPont).

^{14}C-Sucrose Influx Into Soybean Cotyledon Halves at Various Developmental Stages. Soybean pods were harvested at 9, 14, 16 and 18 d after the average flowering date for the population. Cotyledons were removed from the pods and seed coats, the embryonic axis was broken off and the two cotyledon halves were placed in separate incubation containers. Influx of 0.25 mM [^{14}C]sucrose into the cotyledon halves was measured as described by Thorne (26). The preincubation buffer for one set of cotyledon halves contained 15 μM FCCP, while the other did not. Radioactivity in the washed cotyledons after the influx period was determined by liquid scintillation counting after solubilization of the cotyledons which were crushed in NCS tissue solubilizer (Amersham) and heated at 60°C until the tissue had cleared.

Purification of the 62 kD Cotyledon, Membrane Protein. Immature soybean seeds were removed from pods of plants at 18 to 22 DAF and a microsomal preparation was made without removal of the seed from the seed coat. About 60 g of seed were processed as described above with the following modifications: the grinding buffer to seed ratio was increased to 2:1 and the buffer was made to 0.5 mM in phenylmethylsulfonyl fluoride by addition of 50 mM phenylmethylsulfonyl fluoride in isopropanol just prior to grinding. The initial microsomal pellet was obtained by centrifugation of the 13,000*g* supernatant of the grind at 85,000*g* for 50 min. The pellet was washed three times by resuspension in 10 mM Mes-KOH, 2.5 mM DTT at pH 6.8, followed by centrifugation as before. The resulting, washed microsomal pellet was suspended in 100 mM K-PO_4 buffer at pH 7.5 to a protein concentration of 4 to 5 mg/mL and stored in liquid nitrogen until further processing.

Differential extraction of the 62 kD protein from the microsomal preparation was achieved as follows: The thawed, microsomal suspension was brought to 0.2% in octyl-β-D-glucopyranoside (OG) by addition of 20% (w/v) OG in the phosphate buffer and incubated for 20 min on ice. The extract was centrifuged at 150,000*g* for one h and the resulting pellet was suspended at 7 mg protein/ml in 100 mM K-PO_4, pH 7.5, which was 2% (w/v) in OG. After incubation on ice for 20 min, the mixture was again centrifuged at 150,000*g* for one h to obtain the 62 kD protein enriched, OG solubilized proteins. In initial experiments, 1 M urea was used in place of 0.2% OG for the initial solubilization of proteins other than the 62 kD protein with similar results.

The 2% OG extract was equilibrated in 10 mM Tris (pH 7.5) and 2% OG by gel filtration over G-25 equilibrated in the same buffer. About 10 mL of the resulting protein extract was loaded onto a DEAE-Mono Q FPLC ion exchange column (Pharmacia) at 1 mL/min. The column was washed with 6 mL of the loading buffer followed by 2 mL of 0.1 M NaCl in the loading buffer and bound proteins were eluted with a NaCl gradient from 0.1 to 0.5 M. The NaCl concentration was increased by 20 mM/min during the gradient elution and 1.2-mL fractions of the column effluent were collected. Collected fractions were assayed for protein, and aliquots of fractions through the peak of protein elution were taken for analysis by SDS-PAGE after precipitation of the proteins in 10% TCA, acetone washing, and solubilization as described above.

The 62 kD protein eluted at about 0.25 M NaCl over a span of four fractions. Fractions from the first Mono-Q purification were pooled, equilibrated in the column loading buffer and rechromatographed using the same volumes and gradient as in the first column run. Fractions of 0.5 mL each were collected during the gradient elution and again characterized by SDS-PAGE. Fractions which were pure by the criterion of Coomassie blue staining were combined to give a yield of about 4% of the membrane protein solubilized by 2% OG. Silver staining of the pooled fractions showed several lower M_r contaminants and a contaminant of about 59 to 60 kD which was also visible by Coomassie staining of heavily loaded gels.

Polyclonal Antibodies Against the 62 kD Protein. The pooled fractions from the ion exchange purifications were precipitated in 10% TCA, solubilized in the standard solubilization buffer and 200 to 300 μg were applied to a 0.75 mm, 9% polyacrilamide gel for further purification. After development, the gel was stained without fixation in a solution of 9:1 (v/v) 0.1% Coomassie blue:0.5% Serva blue R both in 50% methanol. The gel was then partially destained with 20% methanol containing 3% glycerol, the center of the 62 kD band was cut from the remainder of the gel, frozen, and then ground to a powder in liquid nitrogen. Antibodies were raised in New Zealand White rabbits by Hazelton Research Products Denver, PA.

Western analysis using the anti-62 kD protein antiserum was performed after transfer to nitrocellulose using a Bio-Rad Transblot apparatus and protocol. The blots were washed with a buffer consisting of 20 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% Tween 20 after transfer and blocked with 1% BSA in the same buffer before color development. Color development was done

using alkaline phosphatase coupled to goat anti-rabbit IgG (Promega) with 5-bromo-4-chloro-3-indoylphosphate and nitro blue tetrazolium as substrates.

Immunocytochemistry and Electron Microscopy. *Specimen Preparation.* Developing soybean cotyledons (20 DAF) were cut into quarters and fixed for 12 h at 4°C in one of the following fixatives buffered with 50 mM Pipes (pH 7.2): 4% (v/v) paraformaldehyde; 2% paraformaldehyde and 1.25% (v/v) glutaraldehyde; 2% paraformaldehyde and 2.5% glutaraldehyde. Some samples from each of the above treatments were postfixed for 0.5 h in 1% (w/v) osmium tetroxide in 50 mM sodium cacodylate buffer. All samples were dehydrated with ethanol and embedded in L. R. White resin. Thin sections (60–80 nm) were cut on a diamond knife and picked up on uncoated nickel grids.

Postembedment Immunocytochemistry. Grids with sections were pretreated with TBST buffer (10 mM Tris, 500 mM NaCl, 0.3% [v/v] Tween 20 [pH 7.2] plus 1% (w/v) BSA for 10 min on a shaker. The grids were then incubated for 1.5 h with antisera to the 62 kD membrane protein diluted 1 to 10 with TBST+BSA. After eight 2 min washes with TBST+BSA, the grids were incubated for 1 h with protein A-colloidal gold congugate (10 nm particle size) diluted 1 to 30 with TBST+BSA. The grids were thoroughly washed by immersion in TBST+BSA (5 min), TBST (2 × 5 min), and glass-distilled water (3 × 5 min). Sections were post stained with 2% (w/v) uranyl acetate and 1% (w/v) aqueous lead citrate, then examined and photographed using an Hitachi 300 transmission electron microscope. Some sections were stained with 10% (w/v) phosphotungstic acid (PTA) in 10% (v/v) HCl for 10 min at room temperature, a procedure used to selectively stain the plasma membrane.

***In Vitro* Translation of Poly(A) RNA from Immature Soybean Cotyledons.** Soybean embryos at about 50 mg fresh weight were removed from pods and frozen in liquid nitrogen. Frozen embryos were maintained in liquid nitrogen, ground to a fine powder while frozen and then extracted by polytron homogenization and fractionated to enrich for total RNA (3). The nucleic acid fraction was enriched for poly A RNA by passage through an oligo-dT celluose column with salt elution of the poly A fraction (8).

In vitro translation was done using a wheat germ system with [^{35}S]methionine, about 5 μg of poly(A) RNA, and the protocol described by Viitanen *et al.* (27).

General. Radiochemicals were obtained from New England Nuclear, [^{14}C]-sucrose was purchased at 600 mCi mmol^{-1} and adjusted to an appropriate specific activity with [^{12}C]sucrose before use. Proteins were determined by a dye binding procedure using reagents obtained from Bio-Rad.

RESULTS

Inhibition of Sucrose Influx into Soybean Cotyledon Protoplasts by 6′-HABS. 6′-HABS was shown to behave as a competitive inhibitor of [^{14}C]sucrose influx into protoplasts isolated from developing soybeans (Fig. 1). The competition presumably results from 6′-HABS acting as an alternate substrate for the carrier and therefore displacing labeled sucrose from the active site of the carrier, although no measurement of 6′-HABS influx was made. The apparent K_i for inhibition of sucrose uptake was 60 to 80 μM depending somewhat on the experiment and type of assay. The double reciprocal plots of the data of Figure 1 (inset) give an average K_i of about 60 μM while treatment of similar data by the method of Henderson (10), indicates that inhibition is strictly competitive and that K_i is about 75 μM (data not shown). Neither glucose nor glucose derivatives which lack bulky groups at C-1 are recognized by the sucrose binding site of the carrier protein (11) so AHB-Gln was not tested for inhibition of sucrose influx.

Photolytic Labeling of Membrane Proteins with ^{125}I-6′-HABS.

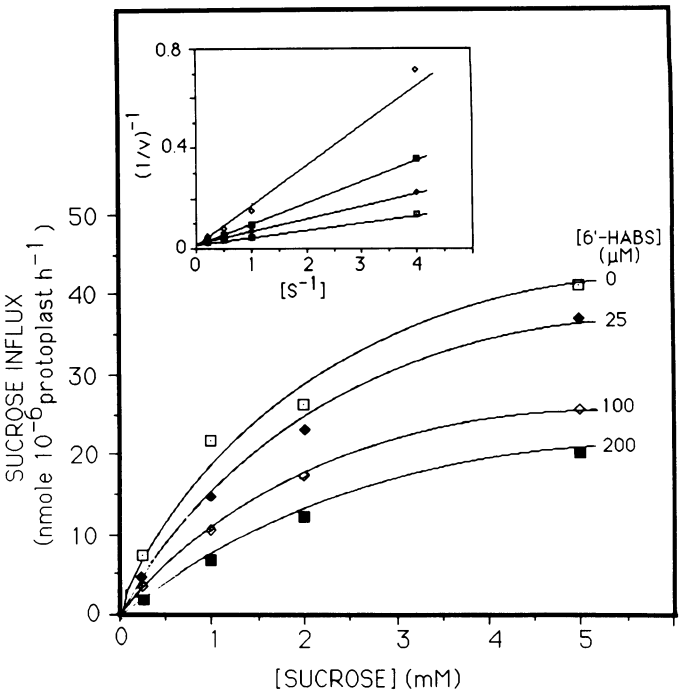


FIG. 1. Inhibition of [^{14}C]sucrose influx into protoplasts from developing soybean cotyledons by 6′-HABS. The K_i for inhibition of sucrose influx calculated from the double reciprocal plot of the data (inset) was 58 μM.

Photolysis of ^{125}I-6′-HABS with high concentrations of membrane protein and DTT present in the photolysis mix resulted in predominant labeling of a moderately abundant protein of about 62 kD by SDS-PAGE analysis (Fig. 2). The pattern of labeling was similar whether photolysis was done at 0°C or at liquid nitrogen temperature (Fig. 2), although photolysis at liquid nitrogen temperature generally resulted in less apparent nonspecific labeling.

The presence of alternate substrates for the sucrose carrier resulted in only partial protection from labeling by 6′-HABS. The dried gel which produced the autoradiogram shown in Figure 2 was cut into segments and counted by gamma counting. In both lane 5 (no protectant) and lane 2 (inclusion of the nonrecognized structure, 3-F-PTG) the band at 62 kD was labeled at 1795 dpm/cm above the average background of the gel lane. Inclusion of 100 mM sucrose in the photolysis mixture (lane 4) reduced labeling of the band at 62 kD to 996 dpm/cm above the average background. The alternate substrate PTG at 5 mM in the photolysis mixture (lane 3) reduced labeling at 62 kD to 1460 dpm/cm. Radioactivity in the gel regions excluding the 62 kD band ranged from 257 to 333 dpm/cm. Protection by 100 mM sucrose thus reduced label incorporation by about 45% while inclusion of 5 mM PTG protected by about 20% in this experiment.

As a check on the specificity of labeling by 6′-HABS, cotyledon membrane preparations were also photolyzed with the ^{125}I-labeled glucose photoprobe AHB-Gln. Photolysis under conditions of probe concentration and specific activity identical to those used with 6′-HABS led to only slight label incorporation into the 62 kD region (660 dpm/cm at 62 kD in lane 1 of Fig. 2), and no identification of other polypeptides.

Sucrose Transport and the Appearance of the 62 kD Membrane Protein during Cotyledon Development. Active sucrose accumulation in cotyledons of different developmental ages was assessed by measurement of [^{14}C]sucrose influx into paired cotyledon halves which were incubated with or without 15 μM FCCP. In the absence of the uncoupler of ATPase, the influx of

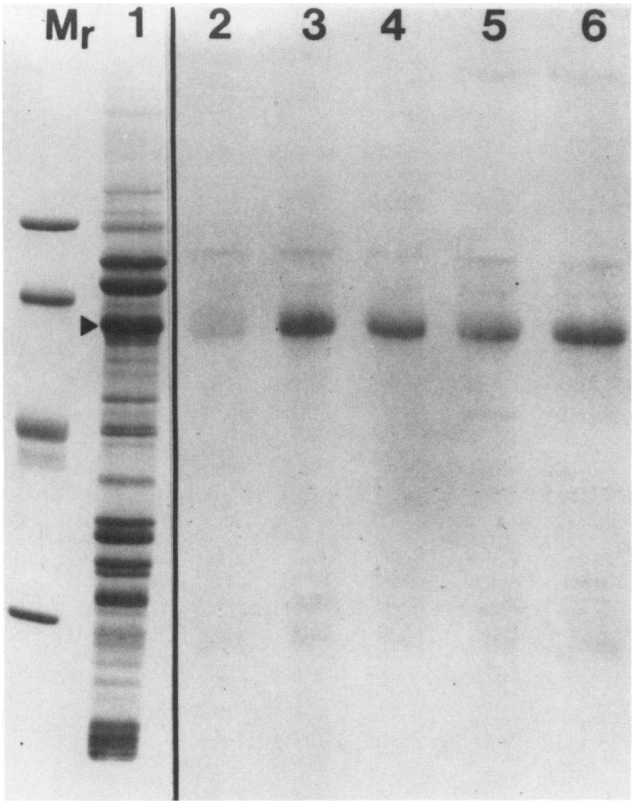


FIG. 2. Photolabeling of membrane proteins from developing soybean cotyledons with ^{125}I-6′-HABS. M_r standards were run in lane M_r and are from the top: 92.5, 66.2, 45, and 31 kD × 10^3. Lane 1 is the Coomassie stain of the dried gel which produced lane 6 of the autoradiogram and is representative of the staining pattern of lanes 2 through 6. Lane 2 is the labeling pattern observed when membrane preparations were photolyzed with the non-substrate probe ^{125}I-AHD-Gln. Lanes 3 through 6 are the labeling pattern observed when ^{125}I-6′-HABS was photolyzed under the conditions described in "Material and Methods." Lane 6 was run without potential protectant, lane 5 contained 100 mM sucrose as protectant, lane 4 contained 5 mM phenyl α-D-thioglucopyranoside and lane 3 contained 5 mM phenyl α-D3-deoxy-3-fluorothioglucopyranoside. When the autoradiogram is overlaid on the coomassie stained gel, the labeled band corresponds to the stained band indicated by the arrow in lane 1.

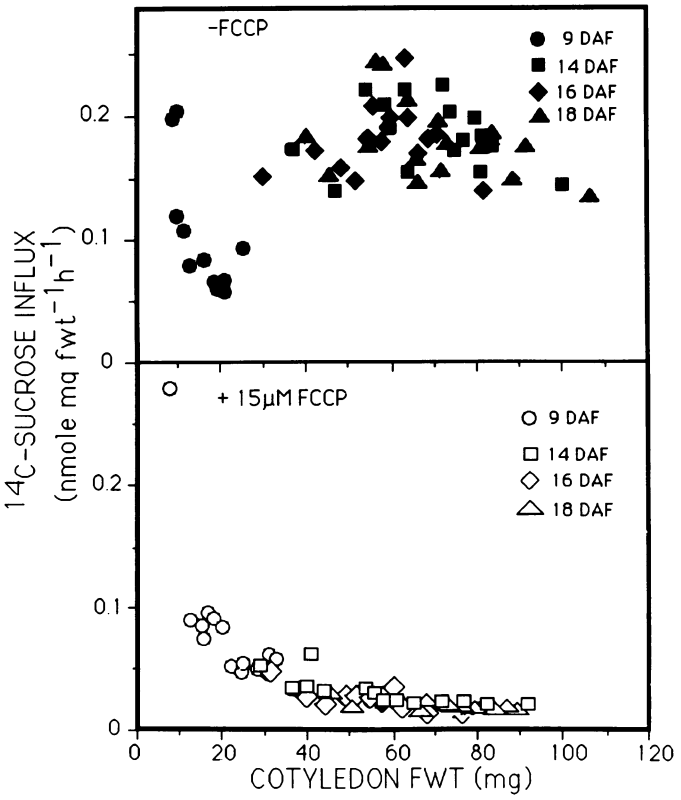


FIG. 3. Influx of [^{14}C]sucrose (0.25 mM) into cotyledon halves at various stages of development. Embryos were split into cotlyedons, and one cotyledon was incubated in aerated buffer for 1 h before initiation of the uptake (top) while one was incubated in aerated buffer with 15 μM FCCP (bottom).

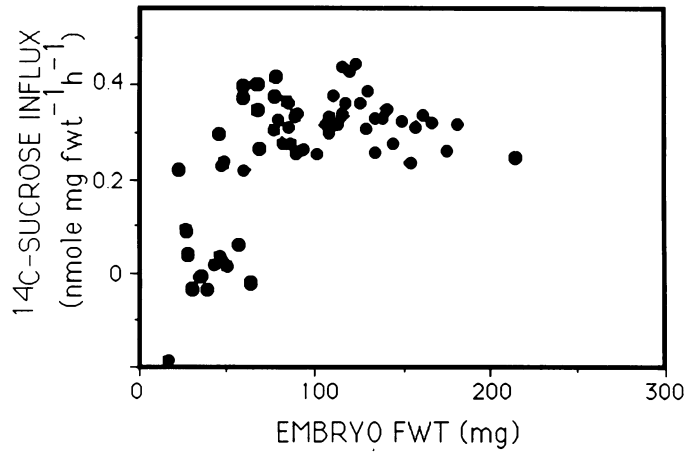


FIG. 4. The estimation of active sucrose influx obtained by subtraction of the FCCP inhibited influx rate from Figure 3 from the noninhibited rate. The difference obtained was doubled to express the influx rate on the basis of the fresh weight of the embryo reassembled from the two halves.

0.25 mM sucrose increased slightly as cotyledon fresh weight and developmental age in DAF increased (Fig. 3, top). When cotyledons were incubated in 15 μM FCCP prior to the measurement of sucrose influx, a sharp decrease in influx was observed as a function of fresh weight and DAF (Fig. 3, bottom) such that influx into cotyledons above about 30 mg fresh weight was severely inhibited by FCCP while cotyledons below that weight were only slightly affected. Figure 4 shows the result of subtracting the influx for the FCCP treated cotyledon half from the influx for the nontreated half, doubling the difference and expressing the rate obtained *versus* the combined fresh weight of the two halves of the reassembled embryo.

Membranes from cotyledons at differing developmental stages were obtained by removal of all pods on plants 9, 11, 13, 16, and 19 after the average flowering date of the plants in the growth chamber at that time for microsomal preparation. Each sampling was thus heterogeneous with respect to cotyledon fresh weight but was representative of cotyledons at times early through late in the time course of the onset of active sucrose accumulation (Fig. 4). Lanes 1 through 5 in Figure 5 are the SDS-PAGE analysis of proteins in the microsomal preparations from cotyledons at the above sampling dates after photolysis with ^{125}I-6′-HABS. The lanes were loaded with equal amounts of protein although the concentration of total membrane protein in fewer components at later ages amy give the appearance of more protein in those lanes.

Several proteins which are prominent in older cotyledons were not detectable by Coomassie staining in the cotyledons at 9 DAF. Among those are the major protein at 62 kD, which appears rapidly over the course of about 5 days. The pattern of protein labeling by photolysis with 6′-HABS shows increased labeling at

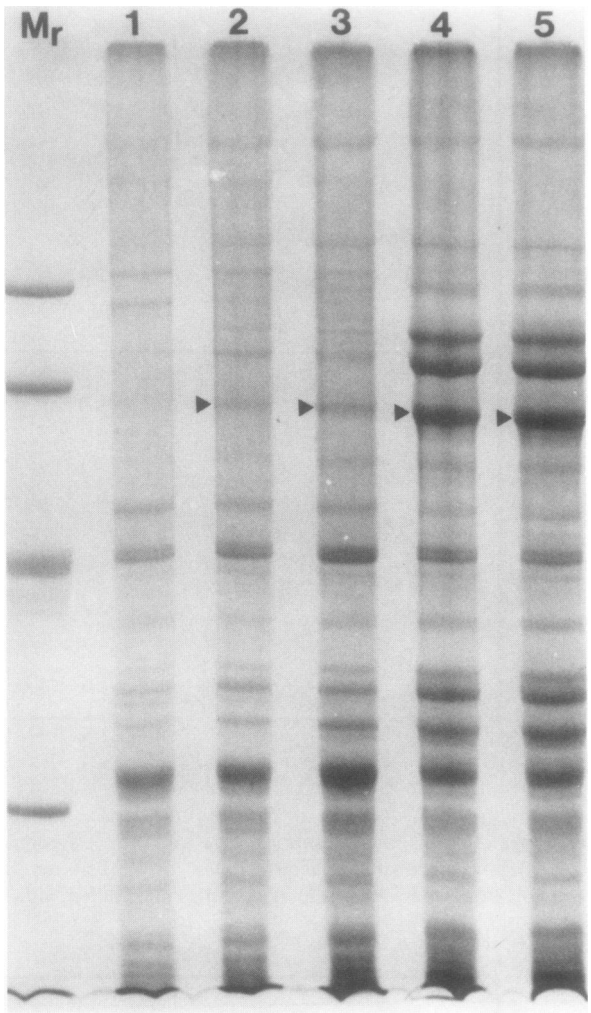


FIG. 5. The Coomassie stained gel obtained from SDS-PAGE separation of the proteins from membrane preparations made from cotyledons at increasing age in days after flowering. Lane M_r as described in Figure 2. Lanes 1 through 5 are the proteins separated from membranes preparations made from cotyledons at 9, 11, 13, 16, and 19 DAF, respectively. Each preparation was photolyzed with ^{125}I-6′-HABS as described in the text before separation.

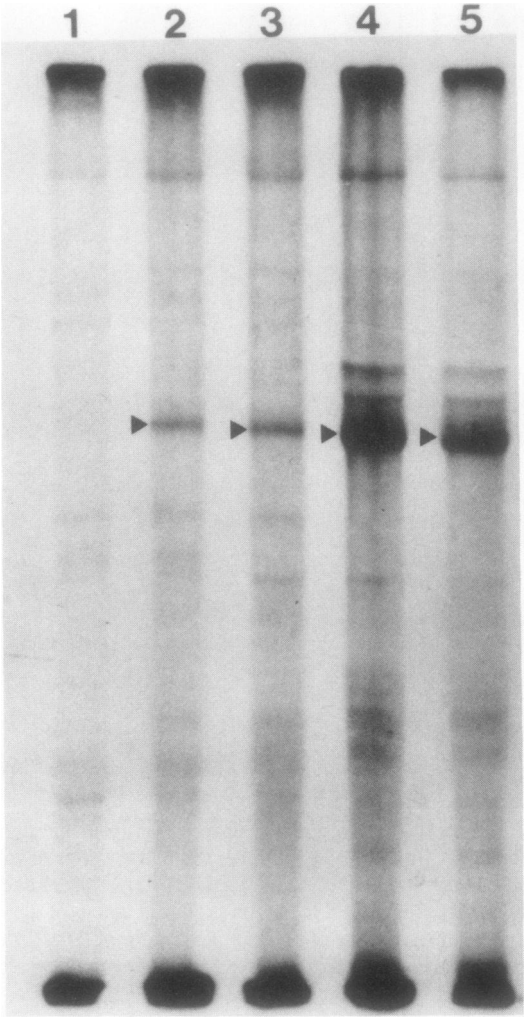


FIG. 6. The autoradiogram corresponding to lanes 1 through 5 of Figure 5. The labeled band marked with the arrow in lanes 2 through 5 corresponds with the stained band marked in the corresponding lanes of Figure 5.

62 kD with the appearance of the Coomassie staining, 62 kD protein (Fig. 6). At 11 and 13 DAF the protein at that position was distinctly labeled although the Coomassie staining protein was very minor.

Purification of the 62 kD Protein. Purification of the 62 kD protein was carried out using SDS-PAGE analysis to assess selection for Coomassie staining proteins at 62 kD at each purification step. Solubilization of some membrane proteins by incubation with 0.2% OG resulted in removal of only a small amount of 62 kD protein from the remaining membrane pellet (Fig. 7), but substantial removal of other proteins such as the band at about 52 kD (Fig. 7, lane 3). Incubation with 1 M urea resulted in virtually the same extraction pattern with somewhat less solubilization of 62 kD protein, and was used in some preparations. Solubilization with 2% OG at the salt concentration used resulted in nearly complete removal of the 62 kD protein from the remaining pelletable proteins (Fig. 7, lane 5), while other proteins such as the multiple bands at about 46 kD were not extracted (lane 4). The resulting solubilized membrane protein preparation contained from 35 to 40 mg total protein when starting with about 60 g fresh weight of immature soybeans and was substantially enriched in the 62 kD protein.

Conditions for 62 kD protein binding to DEAE ion exchange matrices were determined by batch binding to DEAE-sepharose followed by removal of the resin and SDS-PAGE analysis of the nonbound protein. Application of the 2% OG extract at pH 7.5 and low salt concentration to a DEAE-Mono Q column gave complete binding of most proteins while a large amount of pigment and nonprotein, UV absorbing material was eluted in the wash buffers. Pooling of the gradient elution fractions which contained the 62 kD protein, followed by rechromatography gave from 0.6 to 0.9 mg of 62 kD protein which was judged to be at least 90% homogeneous by silver staining of SDS-PAGE gels.

Western Analysis of Cotyledon Proteins Using Polyclonal Antibody Preparations against the 62 kD Protein. Polyclonal antibody to the 62 kD protein immunodecorated only one polypeptide in SDS-PAGE separations from crude homogenates of soybean cotyledons (Fig. 8, lane 5), and the reacting protein corresponds exactly to the purified protein used as the antigen (Fig. 8, lane 2). The supernatant remaining after centrifugation of the crude extract at 89,000*g* was greatly reduced in the amount of immunoreactive protein (Fig. 8, lane 4), while the solubilized pellet from the high speed centrifugation again contained only one reactive protein at 62 kD.

Preimmune serum from two of three rabbits immunized gave no signals in Western analysis of proteins from crude extracts of

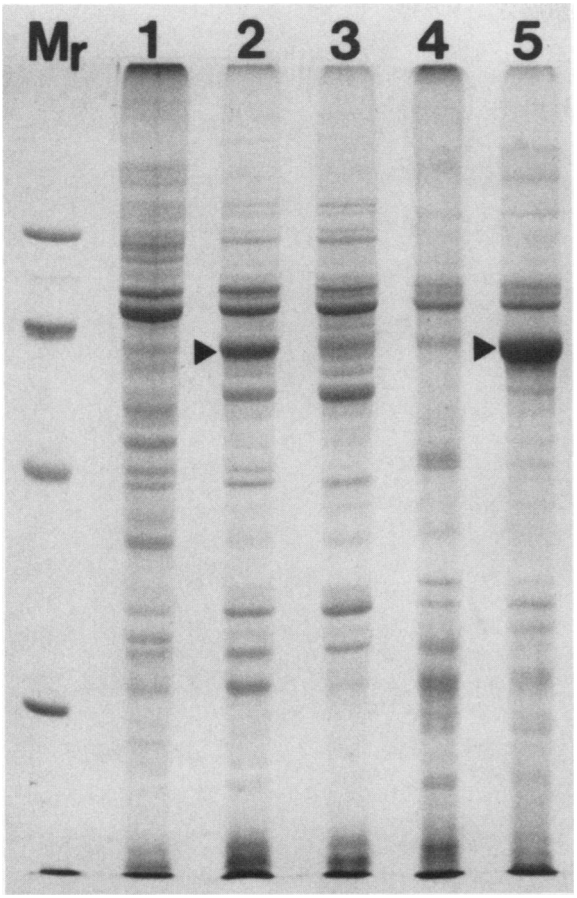


FIG. 7. The partial purification of the 6′-HABS labeled, 62 kD membrane protein by differential detergent extraction. Lane M_r as described in Figure 2. Lane 1 is the SDS-PAGE separation of proteins in the initial grind from the cotyledons. Lane 2 is the protein profile in the microsomal pellet obtained by centrifugation of the initial grind at 150,000*g*. Lane 3 is the protein profile of a 0.1% OG extraction of the microsomal pellet. Lane 5 is the separation of proteins extracted from the pellet obtained after the extraction described in lane 3 with 2% OG, while lane 4 is the profile of proteins remaining in the 150,000*g* pellet after the two, sequential extractions.

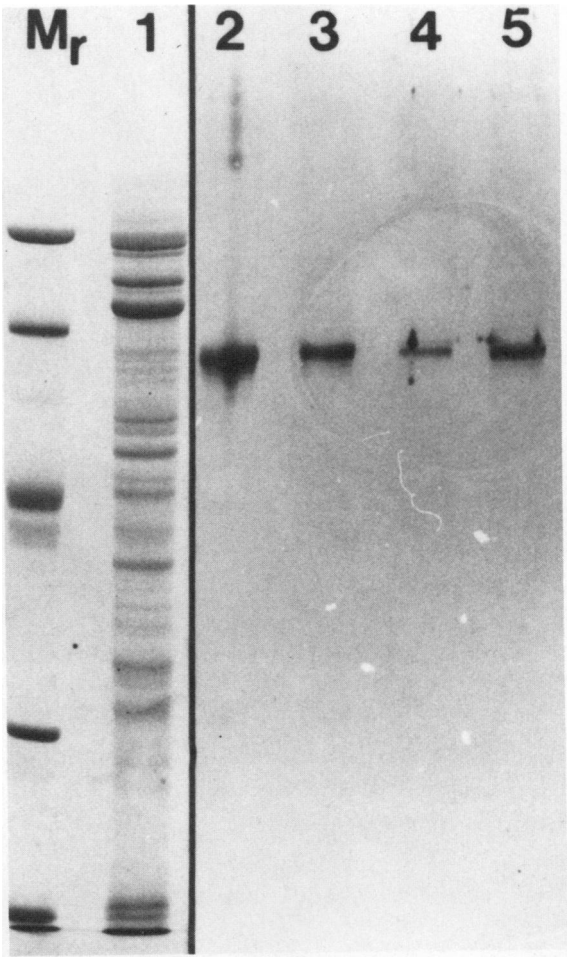


FIG. 8. Western analysis of proteins from developing soybean cotyledons using polyclonal antibody against the purified, 62 kD, membrane protein. Lane M_r shows standards of 92.5, 66.2, 45, 31 and 21.5 × 10^3 kD. Lanes 2 and 3 are two dilutions of the purified, 62 kD protein used as antigen, lane 5 is the Western signal obtained when the total protein extraction (shown in lane 1) is transferred. Lane 4 is the signal from proteins remaining in the supernatant after centrifugation at 89,000*g*.

the immature seeds, while a third serum contained antibody cross-reacting with several proteins, not including the 62 kD protein.

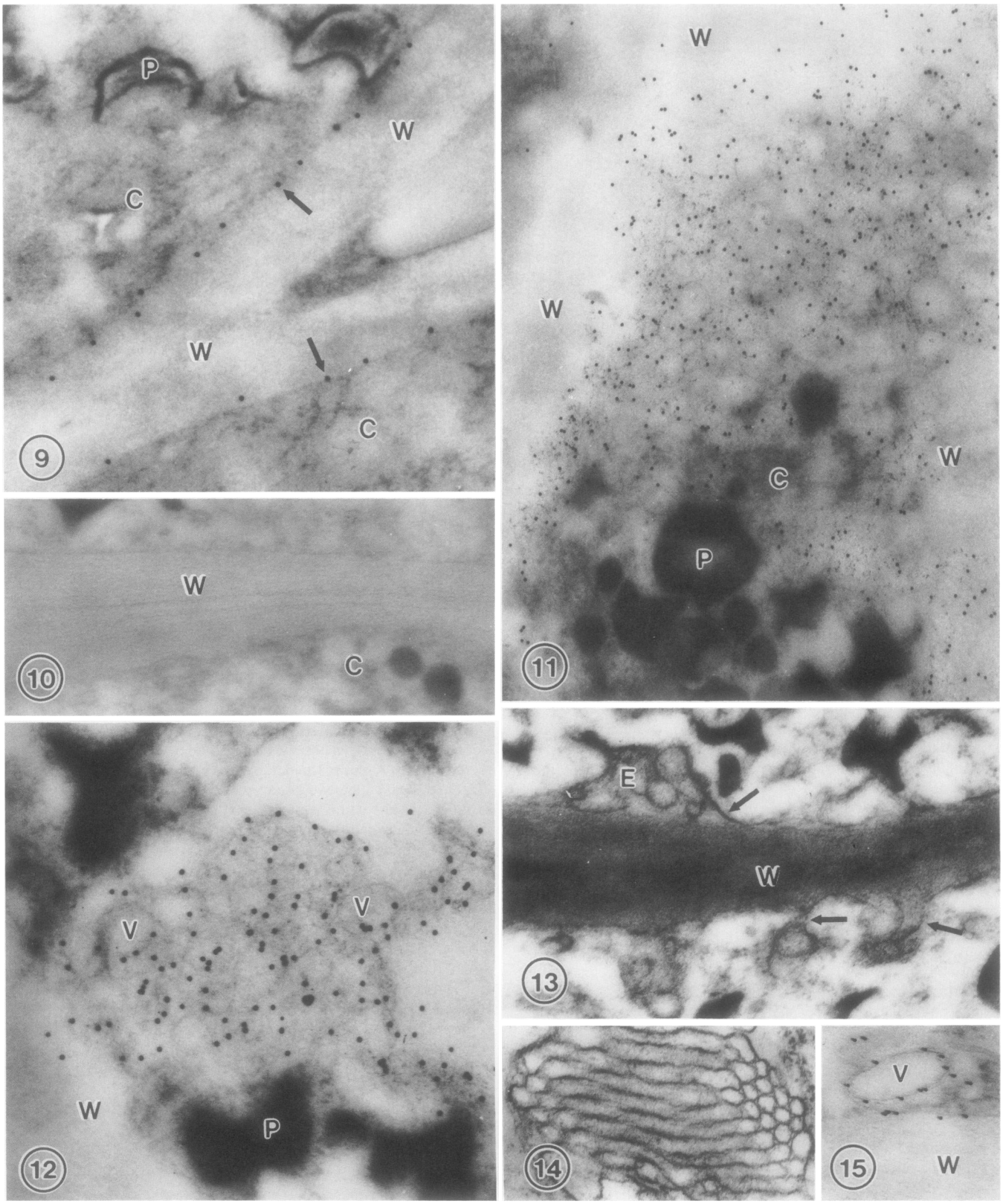
Immunocytochemistry. The immunocytochemical procedure demonstrated that the 62 kD protein is localized along the plasma membrane of cotyledon cells (Fig. 9). All fixation schemes used resulted in effective labeling; however, 2% paraformaldehyde:1.25% glutaraldehyde followed by osmium tetroxide gave the best combination of structural preservation and labeling intensity. No labeling occurred when preimmune serum was used in place of the specific antisera (Fig. 10). Tangential sections along the plasma membrane, giving sheets of membrane within the plane of the section, showed that the protein is abundant and apparently evenly distributed along the plasma membrane (Fig. 11). At no time was any other membrane or organelle seen to be labeled by the antibody preparation.

An interesting observation was that most cotyledon cells contained elaborations of the plasma membrane to form vesicles, tubules, or membrane complexes (Figs. 12–15). The membrane elaborations stained positively with PTA, while other membranes did not stain (Figs. 13 and 14). The plasma membrane elaborations were always heavily labeled by the immunocytochemical procedure (Figs. 12 and 15).

Immune Precipitation of Proteins from *in Vitro* Translation of Immature Cotyledon mRNA. *In vitro* translation of total poly A RNA purified from cotyledons harvested between 15 and 19 DAF using a wheat germ system gave translation products with molecular weights up to about 90 kD as analyzed by SDS-PAGE and fluorography of the [^{35}S]methionine labeled proteins (Fig. 16, lane 2). Treatment of the *in vitro* translate with protein A-Sepharose alone, followed by centrifugation resulted in the precipitation of a small amount of protein typical of the whole translate (Fig. 16, lane 4), while treatment with anti-62 kD serum followed by precipitation with protein A results in a substantial enrichment of a protein which runs very near authentic, purified 62 kD membrane protein (Fig. 16, lane 3).

DISCUSSION

Since benzyl-α-D-thioglucopyranosides are competitive inhibitors of sucrose influx in soybean cotyledon protoplasts with rather low K_i values (11), several photoprobes based on that structure were synthesized. While some were partially successful and in fact had some properties superior to 6′-HABS, none had the required properties of activation at wavelengths above 290 nm and a simple route to radiolabeling at high specific activity with tight binding to the carrier. 6′-HABS competition with



FIGS. 9 to 15. Transmission electron micrographs of 20 d old soybean cotyledon cells immunostained for the 62 kD sucrose binding protein or stained with PTA for plasma membrane (Figs. 13 and 14). Figures 9 and 15 are from nonosmicated samples. Cytoplasm, C; plasma membrane elaborations, E; protein body, P; vesicle, V; wall, W.

Fig. 9. Cross-section through the wall between two cells. The 62 kD protein is located only along the plasma membrane as indicated by gold particles (arrows) (×40,400).

Fig. 10. Section treated with preimmune serum. No labeling occurs (×33,700).

Fig. 11. Tangential cut along the plasma membrane surface showing the density and distribution of the sucrose binding protein on the plasma membrane (×33,700).

Fig. 12. Heavily labeled multivesicular plasma membrane elaboration (×51,000).

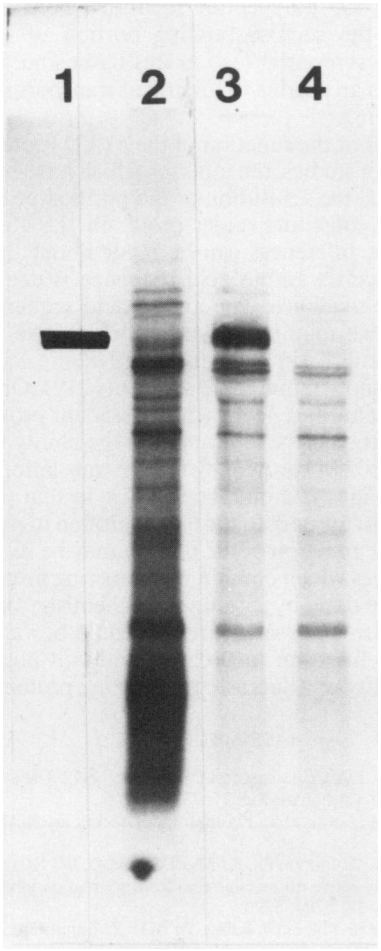


FIG. 16. Fluorogram of the *in vitro* translation products from poly A RNA from soybean cotyledons at about 12 DAF translated in a wheat germ system. Lane 1 shows the position of purified 62 kD protein run on the same gel as the *in vitro* translates. Lane 2 is a separation of the total translate. Lane 3 shows proteins in the precipitate after treatment of the translate with affinity purified, anti-62 kD protein IgG, followed by protein A-Sepharose. Lane 4 is the precipitate obtained using protein A-Sepharose alone.

sucrose uptake was not surprising in view of the hydrophobic nature of substrate-protein interaction at the chosen region of the sucrose molecule (11). The rather low K_i observed was also not unexpected since similar shifts in K_i have been observed from substrates obtained by attaching hydrophobic moieties to other transported sugars (28). The reasons behind this phenomenon are not clear but may be due to a combined interaction of the sugar in its normal binding site and a non-specific binding of the phenylazide in a nearby, hydrophobic domain of the protein.

While the K_i, and presumably k_d for binding of 6′-HABS to the carrier are about 30-fold lower than the k_d for sucrose, a binding constant in the tens of micromolar range is quite high for use as successful affinity probe in systems of mixed membrane proteins (23). Since a comparatively non-abundant protein was expected, photolysis was performed at protein concentrations as high as practically possible, and at photoprobe concentrations as low as possible (*i.e.* at the undiluted specific radioactivity of the probe). This situation should lead to maximal specificity of labeling since specificity is largely a matter of minimizing the formation of nonbound, photoactivated probe (23). At relatively low photoprobe concentrations, a large number of active sites are left unoccupied, but the absolute concentration of unbound probe is very low.

In hindsight, the labeled protein comprises about 3% of the microsomal preparation used in photolysis (given the stated recovery of 62 kD protein from a typical preparation and an assumed recovery of about 30% based on visual analysis of PAGE gels from the fractionation), giving a binding site concentration of about 12 μM. Further assuming that the K_i for 6′-HABS inhibition of sucrose influx is equal to k_d; at 0.57 μM 6′-HABS, the concentration of 6′-HABS:protein complex was about 0.083 μM so that 14.5% of the probe was bound and 0.7% of the active sites were occupied. Lane 5 of Figure 2 contains about 10^{-5} μmol of 62 kD protein (3% of 20 μg). The radioactivity recovered in the 62 kD protein of that lane (1800 dpm) therefore gives a specific radioactivity of about 84 μCi μmol^{-1}. At a probe specific activity of 300 mCi μmol^{-1}, maximal specific activity achievable should have been about 2 mCi μmol^{-1} given the ratio of bound to nonbound active sites. The efficiency of labeling was therefore about 4%.

Two additional steps were taken to maximize selectivity of the probe in combining with sucrose binding sites. First, 10 mM DTT was included as a very effective scavenger of nonbound, photoactivated probe. One of the main reactions of activated aryl azides is nucleophilic attack by amines and sulfhydryls (23). In preliminary tests, several potential scavengers including *p*-aminobenzoic acid, Tris, and other phenyl azides were evaluated. Of these, thiols, including DTT were by far the most effective in reducing label incorporation into proteins other than the 62 kD protein. Since sucrose influx studies with intact protoplasts had also shown binding and uptake to be compatible with, and in fact stabilized by DTT, it was chosen as the scavenger. Secondly, most photolysis experiments were done at near liquid nitrogen temperature after rapid freezing of the photylysis mix. Electron spin resonance spectroscopy after photolysis of aryl azides at 77°K has been used to study trapped triplet nitrenes, and spectral changes in the trapped, reactive intermediates have been observed while still frozen, but at higher temperatures (20). The technique has been applied to labeling of proteins in the reaction centers of photosynthetic bacteria with azido-anthraquinone (20), and azido-atrazine (6), with substantial improvement in specificity of labeling. In labeling with 6′-HABS, photolysis at 0°C led to very substantial labeling of one protein well in excess of its apparent mass as determined from Coomassie staining, but photolysis at liquid nitrogen temperature greatly reduced the smaller amount of labeling of prominent proteins such as those at about 75 and 80 kD (lanes 4 and 5 of Fig. 6 compared to Fig. 2).

Protection from labeling by sucrose or the sucrose analog PTG was never complete, even when the protectant was present in sufficient excess to fully saturate binding. In many photolysis experiments, partial protection was always observed, however, and no protection by structures similar to sucrose or analogs in

FIG. 13. Simple (arrows) and complex plasma membrane elaborations are stained intensely by PTA, indicating they are derived from the plasma membrane (×26,000).

Fig. 14. Micrograph demonstrating that plasma membrane elaborations appear as vesicles or tubules depending upon the plane of the section (×26,000).

Fig. 15. Plasma membrane and an associated vesicle stained positively for the sucrose binding protein. Label is directly on the membrane (×35,000).

binding was seen. For example, 3-F-PTG did not even partially protect from labeling (Fig. 2), nor did the sucrose homolog raffinose (data not shown). The pattern of protection was thus consistent with the sucrose structure being essential for binding although we have no explanation for its being incomplete.

One further check was made on the specificity of 6′-HABS binding. Since the sucrose photoprobe structure contains the hydroxylated phenylazide moiety in addition to the sucrose structure, the possibility existed that the 62 kD protein specifically bound the photomoiety and not sucrose. To eliminate this possibility, the 4-azidosalicamide was attached to glucose to give a non-substrate probe with similar physical properties to 6′-HABS. Photolysis using ^{125}I-AHB-Glcn under conditions identical to those used with ^{125}I-6′-HABS led to marginal incorporation of label into the 62 kD protein thus demonstrating specificity for the sucrose portion of the probe in binding.

Two lines of evidence were developed to show association of the 62 kD protein with sucrose transport. First, it had been known for some time that sucrose influx into cotyledons at very early developmental stages was not coupled to production of a membrane potential (W Lin, C VerNooy, personal communication). This is shown for the tissue used in these experiments in Figures 3 and 4. Uncoupler sensitive sucrose uptake appears rapidly as the cotyledons enter the stage of rapid dry weight increase and accumulation of storage proteins. During this same developmental period, several new proteins appear in microsomal preparations. Among those is the 62 kD protein which is labeled by 6′-HABS. The protein thus appears in a time sequence which is consistent with the process which it is presumed to catalyze. It is also noteworthy that the intensity of labeling closely follows the intensity of Coomassie staining. This, along with copurification of the label and Coomassie staining protein at 62 kD through differential detergent extraction and ion exchange chromatography strongly suggest that the two signals are from the same protein and not simply overlapping by SDS-PAGE analysis.

The second line of evidence comes from the immunocytochemical localization of the protein. If the 62 kD protein functions in the initial steps in sucrose influx into the cotyledon cells, it is most reasonable that it be located in the plasmalemma. That in fact was shown to be the case since immunolabeling in other portions of thin sections of cotyledon cells does not approach the density of labeling of the plasmalemma. Two other observations from these studies are noteworthy. First, the density of immunolabeling on an area basis is quite high when oblique sections through membranes are cut (Fig. 11). This is consistent with the abundance of the 62 kD protein in SDS-PAGE analysis. Secondly, in many sections internal elaborations of the plasmalemma can be seen (Figs. 12–15). Such structures stain histochemically as plasmalemma and have been described by others (2). Since they are often seen associated with cells having a specialized transport function (2, 9) or cells undergoing very rapid growth (14), a role in enhancement of solute uptake has been postulated. These elaborations always immunostain heavily with antiserum to the 62 kD protein (Fig. 12), as might be expected if it is in fact functioning as the importer of the main carbon source for growth.

CONCLUSION

From the above studies it can be concluded that the 62 kD protein identified by photolabeling with 6′-HABS is exclusively a plasmalemma protein. By its detergent solubility properties it seems likely to be an integral membrane protein rather than peripherally associated with the outer membrane. The competition and specificity of labeling studies also suggest that it is a sucrose binding protein, while the timing of its appearance in the plasmalemma is consistent with its playing a role in sucrose influx. The 62 kD polypeptide identified here is thus a good candidate for the sucrose binding portion of the postulated sucrose/proton symporter (18) in this tissue and, if transport in plant systems is analogous to microbial transport (22), the entire transport protein.

Further proof of the function of the 62 kD protein must come from a variety of studies, the tools for which have been developed here. Functional reconstitution of the purified protein into lipid vesicles would constitute direct proof. In the absence of such assays however, inferences can be made about the function of the protein once its amino acid sequence is deduced from its structural gene sequence. The amino acid sequences of several bacterial and two mammalian transporters have been deduced from their structural gene sequences and their calculated secondary structures show remarkable similarity (19). One first step in cloning the structural gene for a nonabundant protein is production of an enriched cDNA library, and the ability to identify the message for the 62 kD protein by *in vitro* translation and immune precipitation (Fig. 16) should be valuable in that process.

Another use of antisera to the 62 kD protein to provide indirect evidence for the function of the protein may be its use to localize specific cell types which contain crossreacting proteins. Whether such studies are done by immunocytochemistry or by cell isolation and Western analysis, the results could be valuable. Studies along all these lines are in progress and will hopefully yield a definitive identity of a sucrose transporting protein in plants.

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