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Sugar uptake by photomixotrophic soybean suspension cultures[1]

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Abstract. A photomixotrophic soybean (*Glycine max*) culture with substantial chlorophyll content was used to study the ability of cells to grow on different carbon sources. With the exception of sorbitol, these photomixotrophic cells were able to grow on media containing 3% starch, or 2% sucrose, maltose, glucose and fructose; and the growth curves and biomass production of hexose- or disaccharide-grown cells were all fairly similar. The highest biomass production was found in the sucrose-grown cells. Levels of soluble protein and total chlorophyll and the Chl a/b ratio were highest in the starch-grown cells. Although the growth curves of sucrose-, glucose-, or fructose-grown cells were quite similar, the abilities of cells grown on one source to utilize various carbon sources were quite distinct. ^{14}C-labelling experiments showed that the highest incorporation rates for sucrose and hexoses all occurred during the logarithmic phase with sucrose-grown cells preferentially taking up sucrose and hexose-grown cells preferentially taking up hexoses. Cells maintained on 3% starch showed an ability to take up both sucrose and hexoses with the highest uptake rate when fed with ^{14}C-glucose. The rate of uptake of sucrose was decreased more than twofold as cells entered the stationary phase, while there was a twofold increase in the activity of extracellular acid invertase (EC 3.2.1.26). The clear correlation between low acid invertase activity and high levels of incorporated radioactivity from ^{14}C-sucrose strongly suggested that intact sucrose is being taken up in sucrose-fed soybean cells during the vigorous growth stage, a stage similar to developing soybean pods.

Keywords: Acid invertase; *Glycine max*; Photomixotrophic suspension culture; Sugar uptake.

Abbreviations: AI, Acid Invertase; **Chl,** Chlorophyll; **PA,** Photoautotrophic; **PM,** Photomixotrophic.

Introduction

There are two known mechanisms for uptake from the apoplast of sucrose in higher plants: One is the direct uptake of exogenous sucrose (for references, see Madore and Lucas, 1989). Alternatively, sucrose is hydrolyzed extracellularly to glucose and fructose, and then retrieved by cells in the form of hexose (for references, Eschrich, 1989). In both cases, transport proteins mediate the uptake of these sugars from the extracellular space through the plasma membrane. Two kinds of carriers, namely sucrose and hexose transporters, have been identified from several plant species (Riesmeier et al., 1992; Sauer et al., 1990). The location of specific carriers in specific tissues/cells is important in the partitioning of sugars between sources and sinks (Riesmeier et al., 1994).

However, how specific cells sense sugars and what the signalling pathways are which induce specific carrier protein are still unknown. Furthermore, determination of uptake kinetics using intact leaves or leaf discs is complicated by the contribution of intercellular invertase and the diffusive resistance of the cuticular layer. To eliminate the factor of intercellular transport, isolated protoplasts have been used extensively (Brown et al., 1997; Fieuw and Willenbrink, 1991) but only for short-term labelling studies. Photomixotrophic (PM) cells offer a system which is photosynthetically active but still requires an exogenous sugar supply and a comparatively homogeneous material for synchronous induction, growth measurements, and uptake studies. Previous studies showed that the physiological state of these cultured cells is most comparable to that of young dividing leaf cells (Rogers et al., 1987). We have maintained a soybean cell line grown on 3% starch as the major carbon source and have investigated the ability of these cells to utilize various exogenously supplied sugars as their carbon source.

Materials and Methods

Cell Culture

The cell line used in this study was originally provided by Dr. J.M. Widholm, University of Illinois. The PM callus of soybean was maintained on solid medium according to Horn et al. (1983). The suspension cells were raised by placing cells in the liquid medium containing 3% starch (Aldrich Co.) and subculturing every 3 weeks. The experiments were initiated by transferring 3 ml of 3-wk-old concentrated cells into 100 ml of liquid medium containing 2% sucrose, glucose, fructose, maltose, or

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sorbitol. Media containing sucrose, glucose, maltose and starch were routinely autoclaved, while those containing fructose and sorbitol were filter-sterilized through 0.45 μm Millipore filter to avoid decomposition. Cultures were incubated at room temperature on a gyratory shaker at 120 rpm and a 16 h photoperiod was provided by Philips cool white fluorescent tubes with a light level of 100 μmol quanta m^{-2} s^{-1}.

Growth Measurements

For fresh weight measurement, the cells were collected by vacuum filtration for 1 min on Whatman #1 filter paper and weighed. Chl was extracted from the cells with 80% (v/v) acetone and measured spectrophotometrically (Arnon, 1949). Contents of soluble protein were determined by Bio-Rad Protein Assay reagent according to the manufacturer's directions (Bio-Rad Laboratories). Concentration of dissolved O_2 in the culture medium was measured using a portable oxygen meter (HI 9143, Hanna Instruments). Rates of O_2 uptake were calculated by subtracting values before and after addition of 1 g of cells into a fresh culture medium for 5 min under light. For the growth curve measurement, cells were grown in an Erlenmeyer flask with a side arm (8 ml). The packed cell volume (PCV) was determined after sedimenting cells in the side arm for 30 min.

Radioactive Labelling

Fifteen ml of cells grown on different sugars was collected at the initial stage (4th), logarithmic stage (8th & 10th), and stationary stage (16th & 21th d after being subcultured). The cells were centrifuged at 4,500 rpm for 10 min, and the supernatant was removed. The fresh weight was recorded, and the cells were then resuspended in 5 ml of fresh medium containing the original sugar. Cells were preincubated for 30 min to allow recovery from transfer shock. D-[U-^{14}C] fructose (276 mCi/mmol), D-[U-^{14}C] glucose (297 mCi/mmol), or [U-^{14}C] sucrose (626 mCi/mmol) (Amersham, UK) was added (1 μCi per tube) separately to cells grown on different sugars. Cells were then incubated under light with gentle shaking. At time 0 and 1 h, cells were collected by centrifuging at 4,500 rpm for 10 min, washed three times with ice-cold fresh medium, and transferred to a scintillation vial. Ten ml of biodegradable aqueous scintillation cocktail (Ecoscint H, National Diagnostics Co.) was added to the washed cells, and radioactivity (dpm) was measured by liquid scintillation spectroscopy. Uptake of sugars was calculated by subtracting the amount of ^{14}C in the time 0 sample from the 1 h sample. Results were expressed as pmol sugar uptake per g tissue per h. All experiments were carried out with three replicates per sample.

Cell Wall-Bound Acid Invertase (EC 3.2.1.26) Assay

Twenty-five ml of suspension cells was incubated in an extraction medium containing 100 mM $CaCl_2$ for 30 min with gentle shaking to release Ca^{2+}-extractable cell wall-bound proteins (Yen et al., 1994). After centrifugation at 4,500 rpm for 10 min, the supernatant (referred to as conditioned medium) was transferred to another tube and used as the enzyme extract after desalting on a 5% (w/v) Sephadex G-25 column equilibrated with 50 mM phosphate buffer (pH 6.9). To determine cell wall-bound AI activity, enzyme extract (100 μl) was incubated with 0.52 ml of 0.6 M sodium acetate (pH 4.5), 0.1 ml of 1.8 M sucrose at 25°C. The reaction was terminated at 0 and 30 min by placing the tubes in boiling water for 10 min. Hexose sugar concentration was determined enzymatically according to Brown and Huber (1987). AI activity was determined by the rate of conversion of sucrose to hexoses during a 30 min incubation. All enzymes used in the AI assay were purchased from Sigma Chemical Co..

Results

Starch-grown PM cells were transferred to media containing 2% sucrose, glucose, fructose, maltose or sorbitol. These cells were able to grow on all of these media with the exception of sorbitol. Growth of soybean PM cells in glucose, fructose, sucrose, maltose, and starch (Figure 1) demonstrates their ability to utilize mono-, di-, and polysaccharides. Growth rates of soybean cells varied depending on the carbon source supplied (Figure 1). There was little difference in growth rates between cells grown on sucrose, glucose, and fructose, with a logarithmic rate of growth occurring between day 5 and 11 after subculture. The growth curve of maltose-grown cells followed a similar trend, but at a slower rate, to the sucrose-grown cells.

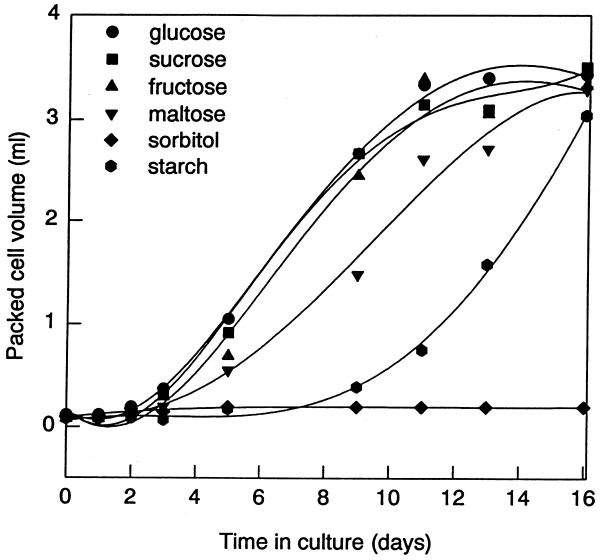

Figure 1. The growth curves of photomixotrophic soybean suspension cells supplied with different sugars. Cells (3 ml) were transferred to media containing 2% glucose, sucrose, fructose, maltose, sorbitol or 3% starch and the packed cell volumes were measured every other day for 16 days. Data are mean values from 2 separate experiments, and each experiment contains two replicates.

The growth curve of starch-grown cells was linear, after an long initial lag (ca. 9 d), during the 16 d test period. Cells maintained on 3% starch never reached the stationary phase in the 16 d test period. In fact, cells were found to grow well on 3% starch up to 2 months and dramatic production of Chl was observed (data not shown).

Several growth characteristics were examined in cells grown in different sugar supplies. Fresh mass, Chl, protein content, and O_2 consumption rate were measured after being subcultured into various sugars and grown for 16 d (Table 1). Maximum fresh mass production occurred with 2% sucrose. Maximum Chl production, Chl a/b ratio and protein content all occurred with 3% starch. However, the level of Chl was only about 20% of that reported for PA cultures (Rogers and Widholm, 1988). The Chl a/b ratios of all treatments (2.03 to 2.32) were lower than that of soybean leaf tissues (ca. 3). Peaks in the absorption spectra for total Chl of starch-grown cells in 80% acetone shifted to shorter wavelengths (425 & 655 nm) compared with those of fully-expanded leaves (432 & 664 nm). Continuous O_2 uptake was observed in all treatments, with the highest value associated with glucose-grown cells and the lowest value associated with starch-grown cells (Table 1). Thus, the amount of O_2 evolved from photosynthesis was not sufficient to compensate for the amount of O_2 consumed by the high rate of cell respiration. Assuming the rate of cell respiration in the light remained relatively constant in all treatments, then the starch-grown cells would have the highest photosynthetic capacity, consistent with their having the highest Chl content.

The continuous O_2 consumption by these highly chlorophyllous cells may be the result of transferring cells to a fresh medium during the O_2 measurement. Different $^{14}CO_2$ incorporation rates were observed when suspension cultures of *Chenopodium rubrum* were incubated in different pH conditions (Hüsemann et al., 1992). However, in the present study, cells continuously consumed O_2 even when the measurements were done in their own conditioned medium under a normal lighting environment (data not shown).

^{14}C-labelling experiments were undertaken to characterize the selectivity of uptake of different sugars. Rates of ^{14}C-labelled sucrose, glucose, or fructose incorporation into cells in different stages were examined. At the stage before cells entered logarithmic growth, i.e. 4 d after transfer, the ability of cells to take up all three sugars was low (less than 10 pmol/g fw/h). As cells entered the logarithmic phase (the 8th day), the rate of uptake of ^{14}C-labelled sugars increased, with sucrose-grown cells preferentially taking up sucrose and hexose-grown cells preferentially taking up hexoses (Table 2). The discrimination between glucose and fructose was not significant in hexose-grown cells (Table 2). Cells maintained on 3% starch showed high incorporation rates of both sucrose and hexoses, with the highest rate when fed with ^{14}C-glu-

Table 1. The growth characteristics of 16-day-old PM soybean suspension cells supplied with 2% sucrose, glucose, fructose, maltose, sorbitol or 3% starch.

Carbon Supply	Fresh Mass[a] (g)	Chlorophyll Content[b] (μg Chl/g fw)	Chlorophyll a/b ratio[b]	Soluble Protein[a] (mg protein/g fw)	O_2 consumption[b] (nmol/ml culture/h)
Sucrose	16.23 ± 0.95	177.26	2.18	8.08 ± 0.43	1.88
Glucose	15.16 ± 1.25	192.26	2.14	8.15 ± 0.64	3.38
Fructose	14.60 ± 1.21	207.19	2.03	9.85 ± 0.52	3.00
Maltose	14.97 ± 2.75	191.99	2.04	7.67 ± 0.43	2.44
Starch	14.32 ± 1.08	312.72	2.32	10.65 ± 0.44	1.73
Sorbitol	1.05 ± 0.16	nd	nd	nd	nd

[a]Means ± SD of three experiments, each representing 2 replicates.
[b]Average of two replicates from one experiment measured in light (100 μmol quanta m^{-2} s^{-1}).

Table 2. Uptake of ^{14}C-labelled fructose, glucose or sucrose by soybean PM cells. Eight-d-old (log phase) and 16-d-old (stationary phase) cells grown in different sugars were incubated separately with ^{14}C-labelled sucrose, fructose or glucose for an hour. Rates of ^{14}C-labelled sugar uptake were expressed as pmol/g fw/h. Data are expressed as means ± SE, N=3.

Carbon supply	^{14}C-sucrose	^{14}C-fructose	^{14}C-glucose
Rate of uptake of ^{14}C-labelled sugar at the logarithmic phase			
Sucrose-grown	95.6±6.0	29.2±7.7	26.2±6.2
Glucose-grown	4.0±2.0	27.7±8.8	38.6±9.9
Fructose-grown	12.5±1.4	31.6±4.2	22.5±2.7
Starch-grown	52.8±5.4	64.9±3.1	104.0±12.3
Rate of uptake of ^{14}C-labelled sugar at the stationary phase			
Sucrose-grown	36.8±4.3	21.6±1.4	5.8±2.9
Glucose-grown	13.3±1.5	12.0±1.3	11.1±3.4
Fructose-grown	27.3±5.7	21.2±4.9	23.1±8.1
Starch-grown	12.8±2.0	15.0±1.8	17.3±4.6

cose (Table 2). Radioactivities incorporated into cells dropped significantly, ranging from 5 to 36 pmol/g fw/h, as growth reached the plateau phase, i.e. the 16th day (Table 2), indicating the possible depletion of carbon source and aging of the cultured cells.

Exogenous acid invertase activity was measured to verify that the high incorporation rate of ^{14}C-sucrose observed in the sucrose-grown cells was not the result of breaking down symmetrically-labelled ^{14}C-sucrose during the one hour labelling period. The activity of Ca^{2+}-extractable (presumably cell-wall bound) AI was low in the 8-d-old sucrose-grown cells, suggesting the majority of ^{14}C-sucrose would remain in the form of intact sucrose (Figure 2). Furthermore, as cells entered the stationary phase (day 16), there was a twofold increase in the activity of extracellular AI that paralleled a threefold decrease in the rate of sucrose uptake (Figure 2). At the end of the third week (day 21), cells were no longer taking up sucrose or secreting AI.

Discussion

Cultured green cells from higher plants offer an attractive system for research at the cellular level for a variety of biological studies (for a review, see Widholm, 1992). Providing suitable light intensity, lowering the sucrose concentration, or replacing sucrose with starch in the culture usually leads heterotrophic cells to develop into PM cells. PM cells have substantial amounts of chlorophyll but still require an exogeneous organic carbon source. PA cells are raised by withdrawing organic carbon from the culture medium and increasing the CO_2 concentration in the culture environment. The photosynthetic characteristics of several PM and PA cell lines have been well-established (Widholm, 1989), and a few PA cell lines can grow in ambient air (350 µl/l CO_2) with a rate of CO_2 fixation comparable to mature mesophyll cells (Goldstein and Widholm, 1990). Unlike PA cells, PM cells have a combination of photosynthetic properties and the capacity to utilize sugars which make them a very useful tool for examining the ability to transport different sugars in plant cells. Since different sugar uptake systems were found in plants (Grimes et al., 1992; Riesmeier et al., 1992; Sauer et al., 1990), a PM cell line of soybean was chosen for study due to its ability to use exogeneous sugars as well as to perform photosynthesis, the physiological conditions closest to young developing pods.

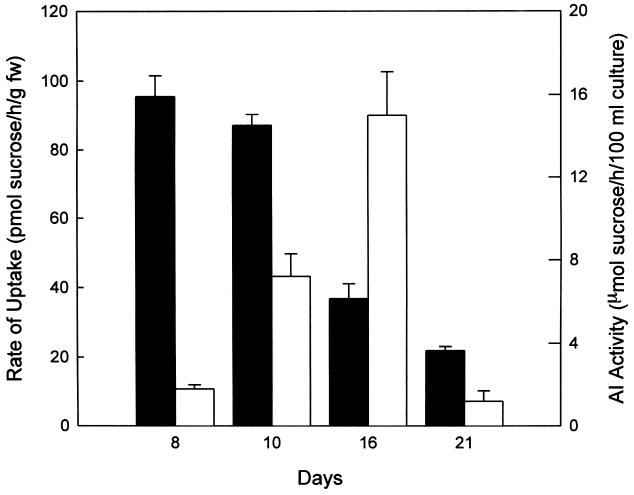

Figure 2. Uptake of ^{14}C-sucrose and cell wall-bound AI activity in sucrose-grown soybean PM cells through the growth cycle. Cells grown in 2% sucrose were harvested at different stages: logarithmic stage (8th & 10th d after subculture), and stationary stage (16th & 21th d after subculture). Cells were used in ^{14}C-sucrose uptake study (black bar, left ordinate) and conditioned media were used in the assays of AI activity (white bar, right ordinate). Data of AI are means from two independent experiments.

Although these cells had been maintained in a starch-containing medium, they grew equally well once transferred to media containing hexoses (glucose & fructose) or disaccharides (sucrose & maltose) indicating that they were able to use various sugars as their carbon source. Growth curves of soybean cells are all typically sigmoidal in hexose- and disaccharide-grown cells, but entry into the logarithmic phase was delayed nevertheless (5 d after subculture) compared with that of dark-grown heterotrophic suspension cells (3 d after subculture when supplied with 3% sucrose). Uptake studies also showed a low rate of ^{14}C incorporation at this initial phase. A reasonable explanation is that, during the initial growth period, energy was provided by the carbohydrate reserves stored in these photosynthetically active cells. As cells enter the logarithmic phase, uptake of exogeneous sugars is necessary to maintain a vigorous growth. Sorbitol, a polyol, is a poor carbon supply for these cells although a sorbitol metabolizing pathway has been found in the axes of germinating soybean (Kuo et al., 1990) and in embryogenic callus of maize (Swedlund and Locy, 1993).

Cells grown in starch as the sole carbon source developed photosynthetic characteristics that are consistent with reports from the original cell line supplier (Horn et al., 1983; Rogers and Widholm, 1988). Theoretically, starch provides a steady but slow release of glucose to the soybean cells. Kim et al. (1997) recently found high α-amylase activity in a light-grown callus of French bean. In this report, we also show the high rate of hexose uptake by these starch-grown cells (Table 2). The slow release of glucose from starch supplied in the culture medium, rather than the direct addition of a high level of glucose to the medium, omits the possible feedback inhibition to photosynthesis by sugars (Sheen, 1994) as we have discussed elsewhere (Yen et al., 1995). Why did these starch-grown cells, though never exposed to sucrose, show an ability to take up sucrose at a high rate in the logarithmic phase (Table 2)? A possible explanation is that sucrose is synthesized and exported by these photosynthetically active cells and gradually accumulates in the culture medium. The sucrose transport system of these cells was then induced in the presence of sucrose (Chiou and Bush, 1998).

There are two known entries of sucrose (the main photosynthate) to developing soybean pods: directly in the form of sucrose or in the form of hexoses after hydrolysis by extracellular AI. Our results indicate that, in the log phase, PM cells of soybean showed a preference for taking up the sugar that was predominant in the culture medium (Table 2). In sucrose-grown cells, the rate of uptake of ^{14}C-sucrose was three times the rate of uptake of ^{14}C-glucose or fructose. Other carbohydrate uptake studies have shown different uptake kinetics when different culture systems were examined. In maize endosperm suspension culture, uptake of ^{14}C-sucrose occurs both by uptake of intact sucrose and by uptake of hexoses generated by extracellular AI (Felker and Goodwin, 1988). In heterotrophic carrot cells, sucrose rapidly disappeared after 3 d, owing to high AI activity, and glucose was preferentially taken up (Kanabus et al., 1985). The cell-wall bound AI activity in our culture system was very low compared to that of heterotrophic carrot cells (Kanabus et al., 1985), was about one-third the cell-wall bound AI activity in developing tomato fruit, and one-fortieth the AI in developing muskmelon fruit (converting the data of Miron and Schaffer, 1991; Hubbard et al., 1989). Therefore, the contribution of AI is considered negligible in the breakdown of the symmetrically-labelled ^{14}C-sucrose during the one-hour labelling period. Indeed, to completely hydrolyze 2% sucrose in the culture medium requires 24 d assuming the average rate of AI activity was 10 µmol/h/100 ml culture.

The sucrose-H^+ symport pathway has been found in developing soybean cotyledons (Lin et al., 1984) and proteins that are involoved in transport or binding sucrose have been reported (Grimes et al., 1992; Riesmeier et al., 1992; Overvoorde et al., 1997). The substrate specificities of various transporters have been examined using heterologous yeast expression systems (Riesmeier et al., 1992; Overvoode et al., 1996); the results were similar to the results obtained by our ^{14}C-labelling experiment (Table 2) with higher uptake specificities between mono- (glucose or fructose) and di-saccharides (sucrose or maltose) and less discriminations within the same group of sugars. In this study, we have shown that this PM culture functions in several respects like a young developing sink tissue. These cells may also bear a resemblance to the mechanism of sugar uptake and serve as a tool for the purification and characterization of the carrier involved in sugar uptake. The PM soybean cells may also offer a homologous system to examine mechanisms and kinetic features of protein-mediated sucrose uptake in plants.

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綠色大豆懸浮培養細胞對醣類之吸收

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本篇是探討綠色大豆懸浮培養細胞吸收不同碳源的能力。此種綠色大豆懸浮培養細胞雖可行光合作用，但仍須培養基提供碳源以維持正常生長。在培養基中加入六種醣類，測試培養細胞的生長情形發現：2% 葡萄糖、果糖、蔗糖、麥芽糖及 3% 可溶性澱粉均可使細胞生長量增加；但 2% sorbitol 則否。生長在單醣及雙醣的培養細胞有相似的生長曲線，但其吸收碳源的種類則不同。比較快速生長期之培養細胞對 ^{14}C-蔗糖、葡萄糖或果糖的吸收速率發現：生長在含蔗糖培養基的細胞，對 ^{14}C–蔗糖有最高的吸收速率，而生長在含葡萄糖或果糖的細胞則分別對 ^{14}C-葡萄糖或 ^{14}C-果糖的吸收率較高。生長在可溶性澱粉之培養細胞，則對此三種 ^{14}C醣類均有很高的吸收率。同時，在快速生長期之蔗糖培養細胞細胞壁之酸性轉化酵素活性很低；推測在快速生長期，生長在蔗糖培養基的大豆懸浮培養細胞是可以吸收蔗糖形式的碳源。上述研究結果顯示，此種培養細胞適合用於研究細胞膜上各種醣類輸送蛋白的分子機制。

關鍵詞：酸性轉化酵素；大豆懸浮培養細胞；醣類吸收。