

***In vitro* Sucrose Uptake and Starch Synthesis by Isolated Wheat (*Triticum aestivum*) Grains**

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ABSTRACT

The aim of this study was to establish and optimise a simplified method of halved grain culture, in order to utilise it to evaluate the ability of isolated sinks (developing wheat grains) in sucrose uptake and conversion (defined by sink strength) under unlimited source conditions. Both sucrose uptake and starch synthesis increased linearly over the incubation periods used in this study. A biphasic pattern of sucrose uptake in response to external sucrose concentrations was evident. Starch synthesis also increased curvilinearly with respect to external sucrose concentration. 2,4-dinitrophenol (DNP) inhibited both sucrose uptake and starch synthesis. An increase in pH from 6.5 to 8 reduced both sucrose uptake and, to a lesser extent, starch synthesis. From these observations, a metabolically active uptake of sucrose by endosperm cells concomitant with a diffusional influx of sucrose into the endosperm cells were both evident. The relative importance of both the active and diffusional movement of sucrose and the importance of transport across the plasma membrane is discussed in the text.

Keywords: DNP, *In vitro* sucrose uptake, Starch synthesis, Wheat.

INTRODUCTION

Both source and sink strength are the main parameters affecting final yield. Different genotypes may differ in their source and/or sink strength and environmental conditions may affect them differently. There is still a debate whether final yield is determined by sink or source limitations, i.e. it is not clear whether the reduced grain growth under certain environmental conditions, or in certain genotypes is due to reduced sink ability to utilise supply or is due to the reduced ability of the source to provide a supply. The incubation of detached grains (sinks) in a medium provides a means of isolating discrete sinks from different genotypes or from a genotype grown under different environmental conditions and determining their ef-

fects on sink strength under unlimited source conditions.

Ho and Gifford [7] observed that the amount of labelled sucrose taken up by peeled grains in the presence of p-chloromercuribenzenesulphonic acid (PCMBS) was higher than ³H-Polyethylene glycol (³H PEG) which does not penetrate into the cells. The difference between the former and the latter was taken as the diffusional movement of sucrose into the cells. Results of the longterm *in vitro* culture of wheat grains showed that, when the culture medium contained less than about 300 mM sucrose, sucrose equivalent concentration was higher in the endosperm cell than in the medium [6]. It was reported that the conversion of sucrose to starch approaches the maximum level at higher external sucrose concentrations and then either remains constant [3] or decreases immediately [8].

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The inhibitory effect of DNP on sugar accumulation in the leaf tissue of sugar beet has been reported [5]. This effect was taken as an indication of a metabolically active loading of sucrose. In wheat endosperm slices, PCMBs reduced sucrose uptake without penetrating into the cells. However, this inhibitor did not reduce the efflux of label from the tissue and also did not affect sucrose uptake at low temperatures where metabolic steps were virtually eliminated [15]. The inhibition of *in vitro* starch synthesis by different metabolic inhibitors (DNP, Carbonylcyanide *m*-chlorophenylhydraz-one [CCCP], and PCMBs) was previously reported for potatoes [13, 16] wheat [15] and maize [4].

Rijven and Gifford [15] found that an increase in external pH from 6.1 to 8 resulted in a decline in total uptake and conversion in wheat grains, with the effect greater in the former than in latter. Lichtner and Spanswick [9] found that changes in external pH from 5 to 8 did not have any effect on sucrose influx by soybean cotyledons, but an increase in pH from 8 to 9 significantly reduced the sucrose uptake. *In vitro* sucrose uptake by slices of maize grain also did not respond to an external pH ranging from 5 to 8 [4].

The aim of this study was to establish and optimise a simple method to study *in vitro* sucrose uptake and starch synthesis mechanisms in order to utilise it for evaluating and comparing the strength of sinks (developing gains) isolated from mother plants of different genotypes or from plants grown under different environmental conditions.

MATERIALS AND METHODS

Plants (spring wheat, *var. Cadenza*) were grown in 12 cm diameter pots containing a 4:1 mixture of Irish moss peat and grit in a glasshouse in the UK. Supplementary lighting ($300 \mu\text{mol m}^{-2}\text{s}^{-1}$ PAR to the top of the plants) was provided by 400W mercury vapour lamps, extending the daily period of light to 16 hours. A daily mean temperature

was maintained at between 19° and 25 °C. Plants were watered daily to near field capacity and supplied with 1 g l⁻¹ Sangral 211 fertilizer once a week through watering. Tillers and subtending leaves were removed from the plants. The “a” and “b” gains (first and second lowest grains in each spikelet) were isolated from the spikelets in the middle of each spike. The glumes, lemmas, and paleae were then removed and the grains used for incubation (see below). Grains were sampled at 15 to 16 d.a.a and used fresh for incubation.

Experiment 1 (time course)

Eight similar plants were selected, and divided into four groups (replicates) of two plants each. Twenty-five grains were isolated from each pair of spikes, mixed and divided into five groups of five and allocated to five incubation times (3, 6, 9, 12 and 15 hours respectively). Grains (five per assay) were then cut transversely under water with a sharp razor blade and both halves placed immediately in 5cm diameter petri-dishes containing 6 ml of incubation solution and incubated at 25 °C under a fluorescent lamp with occasional shaking. Incubation solutions contained 2.5% sucrose in 25 mM Tris buffer (pH 5.5) labelled with a calculated amount of U-¹⁴C sucrose ($0.2 \text{ pCi } \mu\text{l}^{-1}$, 1.73 mCi mg^{-1} sucrose, Amersham International Plc.) to provide a specific activity of 4000 dpm ($0.0018018 \mu\text{Ci}$) mg^{-1} sucrose in the incubation solution. After incubation, the grains were rinsed under running distilled water for 30 seconds to remove any incubation solution from the surface, blotted dry, placed in 80% ethanol and extracted for a total of 6 hours at 80 °C with three changes of fresh ethanol at two hour intervals. Extracts (Ethanol Soluble fraction, ES) were combined and made to 50mls volume. One ml samples were taken from each extract and added to 10 ml of Ecolite liquid scintillation cocktail in a measuring vial and the radioactivity was measured as described for the ethanol insoluble fraction (EINS). Ex-

tracted grains were dried, weighed and finely ground with a pestle and mortar. Samples (5 mg) of ground material were combusted in an oxygen atmosphere in a closed vial containing 1 ml of B-phenylethylamine as a combustion gas absorber using Micro Mat BF 5010 oxidizer. The absorber from each sample was then transferred into measuring vials into which 10 ml of NE 233 and 1 ml of methylated spirits were added as a scintillation cocktail and counted for activity using a liquid scintillation counter (1211 Rackbeta, LKB). Values for each sample were corrected for background radiation, quenching and concentration. Radioactivity in grains was expressed in terms of disintegration per minute (dpm).

Experiment 2 (substrate effect)

Similar plants were paired and twenty grains were then isolated from each pair, allocated to treatments and incubated as described above, except that various concentrations of sucrose (25, 50, 75, and 100 mM) were used in the incubation solutions and the incubation time was 24 hours. After incubation and rinsing, the grains were placed in 200 ml of ice-cold distilled water and shaken for 90 minutes. This procedure allowed the sugars contained in the apoplast to disperse into the cold water [8]. The radioactivity found in the cold water was considered as apoplastic free space sugar [9]. Grains were analysed for ES and EINS as above.

Experiment 3 (inhibitor effect)

Grains were prepared as described in experiment 2 and allocated to two treatments of control and DNP (1 mM DNP in the incubation solution), known as uncoupler of oxidative phosphorylation, both containing 50 mM sucrose. Incubation and ES and ENS analysis procedures were as in experiment 2.

Experiment 4 (pH effect)

Procedures described in experiment 2 were followed except that incubation solutions contained 50 mM sucrose with different pHs (4, 5.5, 6.5, and 8 respectively) for different treatments. A pH value of 6.5 was considered as the control treatment.

Statistical analysis involved the use of one way ANOVA. Data from all experiments are means of four replicates and means given in the tables were compared using Duncan's new multiple range test.

RESULTS AND DISCUSSION

The highest rate of sucrose conversion into ENS (presumably starch), obtained at the highest external sucrose concentration (100 mM) used in the current study, was 0.627 mg this is equivalent to sucrose grain⁻¹day⁻¹ and is considerably lower than the rate of grain growth on the plant at this stage of growth (1.97 mg grain⁻¹ day⁻¹). The average values reported for sucrose concentration in the endosperm cavity are far below 100 mM [7]. These observations may suggest that (a) *in vivo* apoplastic sucrose concentrations appear to be well below the optimum concentration for starch synthesis and (b) starch synthesis in intact gains is more efficient than that of the *in vitro* system probably because of mechanical damage, osmotic shock or metabolic pool disequilibrium.

Time Course of Uptake and Conversion

Figure 1 illustrates the time course of labelled sucrose accumulated in ethanol soluble (ES) and ethanol insoluble (ENS) fractions along with total uptake. The rate of accumulation into ES and ENS fractions, for each period of incubation, is shown in Table 1. A consistent decrease in the rate of accumulation into the ES fraction with increased incubation time is evident. The initial rapid rate was presumably due to the movement of labelled sucrose into the apoplastic free spaces which was not rinsed out after sampling.



Figure 1. Time course of sucrose accumulation and conversion by halved grains (at 15 day after anthesis) incubated in a medium containing 2.5% (73 mM) labelled sucrose (4000 dpm/mg.sucrose). Data are expressed as dpm per mg Dry weight (A) and dpm per grain (B). Each point is the mean of four replicates. Bars indicate standard error of means. ES, ethanol soluble; EINS ethanol insoluble; TOT, total uptake.

Conversion into the EINS fraction showed a general linear increase over time (Figure 1) intercepting the origin on both grain unit and grain dry weight basis. Thus the rate of diffusion of sucrose from the apoplast into the cells did not limit starch synthesis. However, in contrast to the ES fraction, the rate of incorporation for each incubation period tended to be slightly higher for the longer incubation times (Table 1).

Substrate Effect

Figure 2 presents sucrose uptake and conversion with respect to substrate concentration on both mg dry weight and grain bases. A near linear response of net uptake to substrate concentration of between 25 and 100 mM was observed. However, when extrapolated to 0, the total uptake of sucrose into the grain showed a curved response to sucrose concentration, an apparent saturation kinetic

being observed as the exogenous concentration was raised. The rate of net (total) uptake, expressed as both dpm and equivalent sucrose, for each additional sucrose concentration (i.e. the line slope between each two concentration levels) is given in Table 2. A consistent reduction in the accumulation rate for each additional substrate concentration was observed.

Table 1. The accumulation rate of labelled sucrose into ethanol soluble (ES) and ethanol insoluble (EINS) fractions, expressed as disintegration per minute (dpm) grain⁻¹ h⁻¹, for each period of incubation time (data derived from Figure 1).

Incubation period (h)	ES	% Initial	ENS	% Initial
3	471	100	82	100
6	352	75	70	58
9	283	60	85	103
12	268	57	94	115
15	223	47	93	113
24 ^a	159	34	94	115

^a Data from substrate experiment.



Figure 2. Effect of substrate concentration on total uptake (TOT) of sucrose and its partitioning between ethanol soluble (ES) and ethanol insoluble (EIN) fractions by halved kernels isolated from well-watered plants. Grains were incubated in 20 mM Tris-HCl at pH 5.5 with 25 mM KCl, in the presence of different levels of labelled sucrose (4000 dpm/mg.sucrose) concentration for 24 h at 25°C. Each point is the mean of four replicates each of four grains. AF represents appoplastic free space sugar. Data are expressed on a dry weight (A) and grain (B) basis.

The accumulation of label in the ES fraction was relatively linear at between 25 and 100 mM and followed the same pattern as net uptake (Figure 2 and Table 3). Nevertheless, when the line was extrapolated to 0, a biphasic pattern was obtained (Figure 2), with the highest initial rate of uptake at between 0 and 25 mM.

The conversion of sucrose to the EINS fraction also showed a near linear pattern of increase in response to external sucrose concentrations of between 25 and 100 mM (Figure 2 and Table 3). However, the rate of conversion tended to decline for each additional substrate concentration, particularly at higher levels of sucrose concentration.

Table 2. The rate of net (total) uptake of labelled sucrose for each additional substrate concentration and percentage of initial uptake (data derived from Figure 2).

Sucrose conc. (mM)	dpm.	μ g.sucrose		
	Grain ⁻¹ h ⁻¹	% Initial	grain ⁻¹ h ⁻¹	% Initial
0-25	116	100	30	1200
25-50	69	59	17.5	58
50-75	67	58	14	47
75-100	56	48	10.7	36

Table 3. The rate of uptake (dpm. Grain⁻¹ h⁻¹) into ethanol soluble (ES) and ethanol insoluble (ELNS) fractions for each additional sucrose concentration and the percentage of initial uptake (data derived from Figure2).

Sucrose conc (mM)	AF	% Initial	ES	% Initial	ENS	% Initial
0-25	29.5	100	73.5	100	43	100
25-50	24.5	83	43	58	26.7	62
50-75	19	64	42	57	24.3	56
75-100	23	78	38.7	52	17.9	41

AF:Appoplastic free space sugar.



Figure 3. Sucrose uptake and conversion in the absence (CON) and presence of 1.0 mM 2,4 dinitrophenol (DNP). Incubation conditions were the same as described in the legend to Figure 2 except that the sucrose concentration was 50 mM. Data are the mean of four replicates and are expressed on a dry weight (A) and grain (B) basis. Bars indicate SEM. For abbreviations see Figure 2.

The biphasic kinetics of sucrose uptake *in vitro* has been reported by other research workers [9, 12, 13, 16, 17]. This pattern has been interpreted as diffusion into cells superimposed upon a carrier mediated transport—the linear segment indicating a diffusional control process and the saturable part implying an active uptake. Since labelled sucrose from the free space was rinsed immediately following the uptake period, it would appear that the non-saturable phase should not be seen as diffusion into the free space of the tissue but, rather, represents movement into the cell.

Treatment with DNP inhibited both sucrose uptake and its conversion to EINS (Figure 3), the inhibition being considerably greater (11% control) in the EINS fraction than in the ES (46% control). The percentage conversion, as a proportion of the total, reduced from 28% in the control to 9% in the DNP treatment. The reduced movement of sucrose into the endosperm cells and incorporation into starch resulted in an increase in sugar accumulation (156% of control) in the apoplastic free space.

In fact, DNP inhibited the conversion to a greater extent (88% reduction compared

with that of control) than total uptake (63% reduction relative to that of its control). Assuming that DNP effectively eliminated metabolically-dependent transport processes [15], then the amount of uptake by DNP-treated grains should represent the passive transport of sucrose across the membrane determined by the non-saturable component of the sucrose uptake curve. These observations clearly indicate that the total uptake was not entirely diffusional but, at least in part, governed by an energy-dependent process defined by a saturable component of the sucrose uptake curve. The active transport of sucrose across the endosperm membrane has been reported by Gifford and Bremner [6].

The rate of conversion into EINS fraction tended to decline with a higher sucrose concentration, particularly at higher concentrations. A decline in the rate of conversion at higher levels of sucrose concentration to below optimum levels (i.e. lack of linear response) may be attributed to an osmotic effect of sucrose (the reduced osmotic potential of the cell environment). However, this effect was predominant at sucrose concentrations above the optimum level. The

Table 4. The effect of pH on the rate of sucrose uptake and conversion ($\text{dpm.grain}^{-1} \text{ h}^{-1}$) by halved grains incubated for 24 h in 50 mM sucrose. Each value is the mean of four replicates $\pm$ SEM. Means followed by the same letter are not statistically different ($p=0.05$).

pH	ES	EINS	Total
4	453 $\pm$ 4.4a	180 $\pm$ 9.0a	633 $\pm$ 11.0a
5.5	444 $\pm$ 6.0a	157 $\pm$ 2.4ab	601 $\pm$ 7.6a
6.5 ^a C	442 $\pm$ 6.7 a	178 $\pm$ 7.0 a	620 $\pm$ 9.3 a
8.0	335 $\pm$ 4.4 b	146 $\pm$ 6.6 b	481 $\pm$ 10.6 b
P values	0.000	0.01	0.000

^a Control

Table 5. The percentage of conversion as a proportion of the total (data from Table 4).

pH	ES	% Con- trol	EINS	% Control
4	72	100	28	96
5.5	73	103	27	93
6.5	71	100	29	100
8	70	99	30	103

peaking of the substrate response curve of starch synthesis was attributed to osmotic damage caused by sucrose concentrations in the medium [6].

Conversion to EINS was almost eliminated by DNP. Since there was still noticeable labelled sugar within the endosperm cells due to diffusional movement, thus the inhibition of conversion, must have been due to the general uncoupling of membrane-bound ATPase required for starch synthesis.

Effects of pH

Sucrose movement across the plasmalemma might be affected by the pH of the media or $[\text{K}]^+$ as proton extrusion from the endosperm cells can be exchanged for potassium ions. Tables 4 and 5 depict the effects of proton concentration (pH) on sucrose uptake and conversion. An increase in pH from 6.5 to 8—in other words, a decrease in proton concentration, resulted in a significant reduction in net (total) uptake of sucrose (77% of control). Analysis of ES and EINS fractions indicated that the reduction was more

pronounced in the former than in the latter, suggesting that the reduced total uptake was not due to reduced conversion (i.e. diffusional feed-back effect).

One possible mechanism for sucrose movement across the plasma membrane is thought to be proton-sucrose co-transport, which could be stimulated by low pH [5]. The pH of apoplastic sap obtained from wheat endosperm is comparatively low [2 and 7] compared with that of wheat endosperm cells [2] and typical phloem saps (pH 7.4–8.7) [1 and 10]. This acid condition may be maintained by proton extrusion from either (or both) the vascular tissue or the endosperm cells. Furthermore, the uptake of sucrose by endosperm slices was found to be enhanced by fusicoccin which stimulates proton efflux [15]. Niemietz and Jenner [11] found that endosperm protoplast of wheat grain absorbed sugars by diffusion but, in the case of aleurone protoplasts, both diffusion and saturable carrier mechanisms were evident.

An increase in pH from 6.5 to 8 decreased the absolute amount of conversion. The percentage conversion was not reduced but increased, indicating that the reduction in EINS under high pH levels was not due to a reduced efficiency of starch synthesis, rather it was a consequence of the decreased availability of substrate as a result of reduced total uptake. The results also indicate that the starch synthesis system is more adapted to an alkaline pH than to an acidic environment, despite the uptake system which prefers acidic pH. These two different conditions, indeed, naturally exist in those grains where the pH of the endosperm cells (cytoplasmic pH)—where starch is synthesised—is markedly higher than apoplastic pH where sucrose is taken up [10].

The sucrose concentration of the cavity sap of intact grains is much lower (11–46 mM) [7] than the values recorded for optimum starch synthesis by detached grains [6] or endosperm slices in liquid medium [15]. The diffusional component of uptake from the medium with 50 mM sucrose in this study accounted for only 37% of the total



uptake even though grains were halved and thus the endosperm cell membrane had easier access to sucrose compared with *in vivo* conditions where the pathway of sucrose movement into the endosperm is restricted to the aleurone layer [14]. Thus it may be presumed that the contribution of the diffusional component into the endosperm cell *in vivo* is lower than this value. Under such concentrations, membrane carrier-mediated processes are quantitatively important relative to diffusional movement and may imply that the plasma membrane is a potential control point in the utilization of sucrose uptake by wheat endosperm cells. The lower concentration of sucrose in endosperm cells than in the apoplastic sucrose [8] may imply that transport across the plasma membrane into the endosperm cells of developing wheat grains is a potential control point for the utilization of sucrose and thus a rate limiting step in starch synthesis under normal conditions.

The simple method of halved grain culture in a labelled sucrose solution used in this study appeared to be a useful method of studying isolated sinks for their ability to utilise available substrate. This method can be used to study the sink strength of different cultivars and/or to evaluate the lasting and direct effects of environmental stresses on sink strength to utilise substrates provided by mother plant. This is very important when we wish to evaluate the strength of sinks independent of any source influence, in other words under unlimited source conditions.

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جذب ساکارز و تبدیل آن به نشاسته در دانه های جدا شده گندم در حالت *in vitro*

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چکیده

جذب ساکارز و تبدیل آن به نشاسته در حالت *in vitro* به وسیله دانه های جدا شده گندم در مرحله ۱۵ روز پس از گرده افشانی مطالعه شد. جذب ساکارز و تبدیل آن به نشاسته در طول دوره های زمانی استفاده شده در این آزمایش به صورت خطی افزایش نشان داد. وقتی منحنی های جذب ساکارز و تبدیل آن به نشاسته به نقطه صفر در محور مختصات وصل شدند حالت دو فازی در این دو نوع منحنی مشاهده شد. هر دو فرایند جذب ساکارز و تبدیل آن به نشاسته به وسیله DNP (دی نیتروفنل) بازداري شدند. افزایش در pH از ۶/۵ به ۸ نیز هر دو فرایند را بازداري نمود. اگرچه تبدیل ساکارز به نشاسته کمتر تحت تاثیر قرار گرفت. از نتایج این آزمایش دو نوع جذب فعال و غیرفعال ساکارز توسط سلولهای آندوسپرم مشهود بود. اهمیت نسبی این دو نوع جذب و اهمیت انتقال ساکارز از غشای سلولی در متن مقاله بحث شده است.