Utilization and Transport of Glucose in *Olea Europaea* Cell Suspensions

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Cell suspensions of *Olea europaea* var. Galega Vulgar grown in batch culture with 0.5% (w/v) glucose were able to transport $d$-[14C]glucose according to Michaelis–Menten kinetics associated with a first-order kinetics. The monosaccharide carrier exhibited high affinity ($K_m = 50$ μM) and was able to transport $d$-glucose, $d$-fructose, $d$-galactose, $d$-xylose, 2-deoxy-$d$-glucose and 3-$O$-methyl-$d$-glucose, but not $d$-arabinose, $d$-mannitol or $l$-glucose. $d$-[14C]glucose uptake was associated with proton uptake, which also followed Michaelis–Menten kinetics. The transport of 3-$O$-methyl-$d$-glucose was cumulative (40-fold, at pH 5.0) and the protonophore carbonyl cyanide $m$-chlorophenylhydrazone strongly inhibited sugar accumulation. The results were consistent with the involvement of a monosaccharide: proton symporter with a stoichiometry of 1:1. When cells were grown with 3% (w/v) glucose, the uptake of $d$-[14C]glucose followed first-order kinetics and monosaccharide:proton symporter activity was not detected. The value obtained for the permeability coefficient of hexoses in *O. europaea* cells supported the hypothesis that the first-order kinetics observed in 0.5% and 3% sugar-grown cells was produced exclusively by passive diffusion of the sugar. The results indicate that in *O. europaea* cells sugar levels have a regulatory effect on sugar transport, because the activity for monosaccharide transport was repressed by high sugar concentrations.

Keywords: Monosaccharide transporter — *Olea europaea* (cell suspensions) — Sugar repression — Sugar uptake.

Abbreviations: CCCP, carbonyl cyanide $m$-chlorophenylhydrazone; 3-$O$-MG, 3-$O$-methyl-$d$-glucose; TPP+, tetraphenylphosphonium.

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**Introduction**

In higher plants, source tissues are responsible for producing carbohydrates, namely sucrose, by photosynthetic CO$_2$ fixation, or glucose by starch degradation. Assimilated carbon is loaded into phloem cells symplastically and/or apoplastically to heterotrophic sink organs. In sink tissues, unloaded sucrose is used for carbon and energy or storage and can be taken up directly into cells. Monosaccharides resulting from sucrose hydrolysis by apoplastic invertases are also taken up. Higher plants possess two distinct families of sugar carriers: the disaccharide transporters (DST) that primarily catalyze sucrose transport, and the monosaccharide transporters (MST) that mediate the transport of a variable range of monosaccharides (reviewed by Williams et al. 2000). The MST family consists of at least 26 genes in *Arabidopsis thaliana* and multiple genes have been isolated from other plant species. The kinetic properties of the encoded proteins have been studied mainly by heterologous expression in yeasts or *Xenopus* oocytes and all the transporters characterized so far are energy-dependent H$^+$-symporters (Büttner and Sauer 2000). Analysis of expression shows that plant MSTs are highly regulated, such as in response to pathogen attack or after wounding (Lalonde et al. 1999, Delrot et al. 2000, Delrot et al. 2001). The mechanisms underling regulation by sugar levels are not well understood. While in the unicellular green-algae *Chlorella* and in *Vitis vinifera* berries hexose carrier genes are induced by the substrate (Tanner 1969, Fillion et al. 1999), genes encoding hexose transporters in *Chenopodium rubrum* are constitutively expressed and not regulated by the sugar (Roitsch and Tanner 1994). In spite of recent advances in the identification and cloning of genes encoding plant monosaccharide transporters, the physiological and biochemical characterization of carrier proteins using plant cell systems has been difficult. In part, this may be due to the complex sugar transport kinetics often observed, involving one or two saturable phases and a non-saturable linear component (Delrot et al. 2000).

*Olea europaea* L. is one of the most important and widespread crops of the Mediterranean basin. Most of the olive production is destined for olive oil; however, a considerable part of olive production is processed to different types of olives for direct human consumption. Sugars are the main soluble components in olive tissues and play important roles, providing energy and acting as precursors for olive oil biosynthesis. Glucose, fructose and galactose are the main sugars found in olive pulp but appreciable quantities of mannitol are also present (Marsilio et al. 2001). Here we report the characterization of glucose transport mechanisms in cell suspensions of *O. europaea*. Evidences for the involvement of an H$^+$-dependent monosaccharide transporter on glucose uptake are provided, and the results showed that high sugar levels seem to repress carrier expression and/or activity. As 3-$O$-methyl-$d$-glucose (3-$O$-MG) promoted repression of the permease activity, a signal mediated by a plasma membrane sugar sensor/transporter seems to be involved in the sugar-sensing mechanism.

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**Results**

**Growth in batch cultures with glucose**

Cell suspensions of *O. europaea* were tested for their capacity to use sucrose, lactose, glucose, galactose, fructose, mannitol and glycerol, as sole carbon and energy sources. Among these substrates, only lactose and glycerol were not able to promote cell growth (not shown). Growth of cell suspensions with 0.5% and 3% glucose, and sugar disappearance from the medium are depicted in Fig. 1. As can be seen, the exponential growth phase was longer when cells were grown with 3% glucose, and glucose disappeared from the medium within 7–25 d, depending on the initial sugar concentration. The maximum specific growth rates ($\mu_{\text{max}}$) were of the same order of magnitude with both sugar concentrations: 0.07 d$^{-1}$ for 0.5% glucose-grown cells and 0.11 d$^{-1}$ for 3% glucose-grown cells. The utilization of glucose by heterotrophic plant cell suspensions requires its transport across the plasma membrane, which, in turn, may constitute an important step for the control of cell growth. The following experiments were designed to study glucose transport and its regulation.

**Transport of glucose in cells grown with 0.5% glucose**

To study glucose transport, 0.5% (w/v) glucose-grown cells were harvested after 6 d in culture, corresponding to a glucose concentration in the medium ≤0.02%. Fig. 2A depicts results of the initial uptake rates of $\delta$-$[^{14}\text{C}]$glucose, at pH 5.0, over a concentration range of 0.02–50 mM. A biphasic uptake curve was observed and the application of a computer-assisted non-linear regression analysis (GraphPad software, San Diego, CA, U.S.A.) to the data suggested the involvement of a saturable transport system associated with first-order kinetics. The linear component of total glucose uptake was more evident for $\delta$-$[^{14}\text{C}]$glucose than for other sugars (Fig. 3). The results showed that $\delta$-fructose, $\delta$-galactose and $\delta$-xylose behaved as competitive inhibitors, indicating that they share the same carrier (Fig. 3A). According to this figure, the affinity of the monosaccharide carrier for the sugars was in the order fructose < xylose < galactose. $\delta$-arabinose and $\delta$-manni-
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Fig. 3 Eadie–Hofstee plots of the initial uptake rates (pH 5.0) of \[^{14}C\]glucose in cultured cells of *O. europaea* grown with 0.5% (w/v) glucose. The initial velocities were measured in the absence of other sugars (closed diamonds) and in the presence of 10 mM fructose (closed circles), 5 mM xylose (open circles), 1 mM galactose (closed triangles), 5 mM arabinose (open diamonds), and 5 mM mannitol (open triangles) (A) or in the presence of the following glucose analogs: 8 mM 1-glucose (open squares), 0.5 mM 3-O-MG (open triangles) or 0.5 mM 2-deoxy-\(\alpha\)-glucose (closed squares) (B).

Phosphonium (TPP\(^+\)) on the initial uptake rates of 0.02–0.2 mM \[^{14}C\]glucose. TPP\(^+\) inhibited sugar uptake, indicating that membrane potential has a significant contribution to the driving force for sugar transport by the monosaccharide carrier.

To study the accumulative capacity of the carrier, the non-metabolizable glucose analog 3-O-MG was used, since, as shown in Fig. 3B, it is a substrate for the monosaccharide carrier. Fig. 5B shows the transport of 3-O-methyl-\[^{14}C\]glucose, at pH 5.0 and pH 7.0. 3-O-MG was accumulated to levels of about 40-fold and 10-fold at pH 5.0 and pH 7.0, respectively. In addition, the protonophore CCCP prevented 3-O-MG accumulation (Fig. 5B).

**Transport of glucose in cells grown with 3% glucose**

To study glucose transport in suspension-cultured cells grown with higher glucose concentrations, 6-day-old cells of *O. europaea* were grown with an initial glucose concentration of 3%. As shown in Fig. 1B the corresponding glucose concentration in the culture medium was about 2%. Under this experimental condition cells also exhibited the capacity to transport glucose, and the initial uptake rates of 0.02–0.2 mM \[^{14}C\]glucose, at pH 5.0, are presented in Fig. 2B. Analysis to the data was consistent with a linear relationship between the rate of uptake and the sugar concentration, and a value for \(k_d\) of 0.34±0.004 \(\mu\)l min\(^{-1}\) mg\(^{-1}\) DW was obtained. It appears that no carrier-mediated glucose transport system was present in cells grown with 3% glucose. After addition of 0.0025–2 mM glucose to weakly buffered cell suspensions no alkalization sig-
Glucose transport in O. europaea cell suspensions

Nals were detected (not shown), suggesting that proton movements were not associated with glucose uptake by glucose sufficient cells. O. europaea cells grown with 3% glucose were also used to evaluate the transport of 3-O-[14]C-MG along time (Fig. 5B). At pH 5.0, labeled 3-O-MG did not accumulate in cells beyond diffusional equilibrium. Taken together, the results suggest that the H⁺-dependent monosaccharide transporter present in cells grown with 0.5% glucose is not operational in cells grown with 3% glucose until depletion of glucose from the culture medium.

**Transport of glucose in cells grown with sucrose and mannitol**

O. europaea cells grown with 3% sucrose and 3% or 0.5% mannitol were also tested for the capacity to transport D-glucose. In 3% sucrose-grown cells, capacity to invert the sugar was observed, and a glucose concentration in the medium of about 1% was measured in 6-day-old cultures (not shown). Similar to the results obtained for 3% glucose-grown cells, 3% sucrose-grown cells did not display activity for a mediated transport system for glucose, and the sugar was absorbed according to first-order kinetics ($k_d = 0.57 \pm 0.02 \mu l \text{min}^{-1} \mu g^{-1} \text{DW}$) (not shown). The same result was obtained when glucose transport was measured in cells of O. europaea grown with 3% mannitol, a value for $k_d = 0.21 \pm 0.003 \mu l \text{min}^{-1} \mu g^{-1} \text{DW}$ being obtained. However, activity for an H⁺-dependent monosaccharide transporter was recovered when cells were grown with 0.5% mannitol. The following values for the kinetic parameters were obtained: $K_m = 67 \pm 30 \mu M$ glucose; $V_{max} = 1.45 \pm 0.26 \text{nmol glucose min}^{-1} \mu g^{-1} \text{DW}; k_d = 0.1 \pm 0.008 \mu l \text{min}^{-1} \mu g^{-1} \text{DW}$.

**Simple diffusion of glucose**

In addition to passive diffusion at least two alternative possibilities, or a combination of them, could account for the apparent first-order kinetics measured in cells grown in all conditions tested: the occurrence of a channel or a glucose trans-
porter of very low affinity. We have investigated the possibility of the involvement of free diffusion of glucose by determining the permeability coefficient of hexoses in *O. europaea* cells. For such studies, L-glucose was used, since (i) there is no information in the literature reporting the existence of glucose transporters exhibiting activity for L-glucose, and (ii) initial uptake rates of L-[14C]glucose and D-[14C]glucose in membrane vesicles prepared with *E. coli* phospholipids were equal and linearly related to the external sugar concentrations (Gerós et al. 1999). Fig. 6 shows the results of the initial uptake rates of 0.02–50 mM L-[14C]glucose, at pH 5.0, in *O. europaea* cells grown with 0.5% and 3% glucose. The data show a linear relationship between the rate of uptake and sugar concentration. The values for diffusion constants were as follows: for 0.5% glucose-grown cells \(k_d\), 0.52±0.01 μl min⁻¹mg⁻¹ DW and for 3% glucose-grown cells \(k_d\), 0.53±0.02 μl min⁻¹mg⁻¹ DW. These values were similar to the values of \(k_d\) determined for D-[14C]glucose uptake in 0.5% and 3% sugar-grown cells, indicating that the linear component of D-glucose transport is due to passive diffusion of the sugar.

Ethanol and other alkanols interfere with membrane transport in multiple ways: increase of the membrane permeability in the case of simple diffusion; decrease of the capacity of both facilitated diffusion and active transport, probably by interference with transport proteins and/or their lipid environment (Van Uden 1989). For *O. europaea* cells, 4% ethanol inhibited up to 70% the initial uptake rates of 0.02–0.2 mM D-[14C]glucose (saturating component) and stimulated by about 50% the initial uptake rates of 20–50 mM D-[14C]glucose (linear component). These findings support the hypothesis that glucose uptake by these cells consists of mediated transport and free diffusion of the sugar, respectively.

**Regulation of the monosaccharide symporter activity by sugar levels**

The results presented above suggested that the activity of the monosaccharide/H⁺ symporter of *O. europaea* is repressed by high sugar levels in the culture medium, as permease activity was only measured in cells grown with low sugar supply. To demonstrate the induction of transport activity in response to glucose concentration the dependence of the permease activity on sugar levels in the medium was evaluated. Cells were grown in a medium with 0.5% glucose and the uptake of 0.02–0.2 mM D-[14C]glucose was measured in aliquots of the cell culture at

**Table 1** Repression of *O. europaea* monosaccharide/H⁺ symporter by different sugars

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Repression (%)</th>
</tr>
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<tbody>
<tr>
<td>D-glucose</td>
<td>83</td>
</tr>
<tr>
<td>D-fructose</td>
<td>84</td>
</tr>
<tr>
<td>D-galactose</td>
<td>80</td>
</tr>
<tr>
<td>D-xylose</td>
<td>80</td>
</tr>
<tr>
<td>D-arabinose</td>
<td>90</td>
</tr>
<tr>
<td>3-O-methyl-D-glucose</td>
<td>91</td>
</tr>
<tr>
<td>2-deoxy-D-glucose</td>
<td>92</td>
</tr>
<tr>
<td>D-mannitol</td>
<td>84</td>
</tr>
<tr>
<td>Sucrose &quot;</td>
<td>15</td>
</tr>
</tbody>
</table>

The results were obtained 7 h after the addition of the sugar to cultured cells exhibiting maximal carrier activity.

" Hydrolysis of the sugar was not detected.
the time periods indicated in Fig. 7. As can be seen, once the external levels of glucose fall below 0.02% (day 5), the monosaccharide carrier is induced. The maximal activity of the permease was observed after day 6, when glucose was completely exhausted from the culture medium. When a pulse of 3% glucose was added, a markedly decrease of the permease activity occurred, reaching residual levels within 12 h. To study sugar specificity for permease repression, the activity of the permease was measured after a pulse of other mono- and disaccharides. The results are shown in the Table 1, in terms of percentage of permease repression, 7 h after the sugar addition. All the monosaccharides tested, including those which were not recognized by the glucose carrier, such as arabinose and mannitol, promoted repression of the permease activity.

Discussion

In the study reported here we have investigated the way in which glucose is transported across the plasma membrane of *O. europaea* cells and the studies were performed using heterotrophic cells suspensions as a biological model. Our results showed that these cells are able to take up D-glucose by a saturating transport system with high affinity and a non-saturating component with a linear dependence on labeled glucose concentration. When cells were grown with 0.5% glucose or 0.5% mannitol, both modes of glucose transport were measured. When cells were cultivated with 3% sugar (glucose, sucrose or mannitol), and used in transport experiments before sugar depletion from the medium, D-glucose was taken up according to a non-saturating mechanism.

Glucose transport according to non-saturating mechanisms has also been reported in other plant cells and tissues (Delrot et al. 2000, Krook et al. 2000). However, this diffusion-like component has been frequently misinterpreted due to complex kinetics observed for sugar transport in most plant cell systems. In this report, in order to evaluate if free diffusion of the sugar is the only mechanism responsible for the observed first-order kinetics, we have measured the permeability coefficient of hexoses in *O. europaea* plasma membrane, according to that described by Gamo et al. (1995) for the yeast *Saccharomyces cerevisiae*. The authors concluded that the “low-affinity component” of D-glucose transport in this yeast is indeed not due to a passive diffusion since the permeability coefficient of hexoses, measured as the rate of 1-glucose uptake, was at least two to three orders of magnitude lower than required to account for the measured D-glucose uptake. In contrast, for *O. europaea* cells grown with 0.5% or 3% sugar (glucose, sucrose or mannitol), the values of \( k_p \) for D-glucose uptake were similar to those for 1-glucose uptake. These results, together with those regarding the effect of ethanol on D-[\(^{14}\)C]glucose uptake, demonstrate that the linear component of glucose uptake is due to free diffusion of the sugar, and suggest that this is the only mechanism responsible for glucose uptake in glucose sufficient cells. Furthermore, in cells grown in these conditions proton movements associated with D-glucose uptake were not detected and the cells were not able to accumulate 3-O-MG, indicating that an H\(^+\)-dependent monosaccharide carrier was not operational.

The saturating transport system observed in *O. europaea* cells grown with low sugar concentration, appeared to involve a H\(^+\)-dependent monosaccharide carrier with a stoichiometry of 1 glucose : 1 H\(^+\). This conclusion was supported by the following: (i) glucose uptake was associated with a transient alkalinization of the extracellular medium, (ii) initial velocities of proton disappearance followed Michaelis–Menten kinetics and were similar to the initial velocities of 1-[\(^{14}\)C]glucose uptake, (iii) the carrier exhibited accumulative capacity, an accumulation ratio of 3-O-MG up to 40, at pH 5.0, being obtained, (iv) the accumulation ratio was dependent on extracellular pH, and (v) the abolishment of transmembrane proton-motive force by the administration of the uncoupler CCCP significantly inhibited initial velocities of D-glucose uptake and 3-O-MG accumulation. Additionally, D-glucose transport was sensitive to TPP\(^+\) suggesting that the Δ\(\Psi\) is an important component of proton-motive force involved in glucose transport and accumulation. To evaluate the real accumulative capacity of the monosaccharide carrier and the relative weight of Δ\(\Psi\) and ApH on glucose uptake, and to further study the transporter protein at a molecular level, we have initiated studies involving the reconstitution of the carrier activity in liposomes with the enzyme cytochrome c oxidase co-reconstituted as an artificial proton pump (Gerós et al. 1996).

Our results are in accordance with what is described for other types of plant cells where most co-transporters utilize the electrochemical gradient of protons generated by the H\(^+\)-ATPase to drive the transport of substrates across the plasma membrane. The presence of monosaccharide transporters with \( K_m \) values of the same magnitude as that in *O. europaea* cells are described in cells of *Nicotiana tabacum* (Verspagen et al. 1991), cell suspensions of *Daucus carota* (Krook et al. 2000), in guard cell protoplasts of *Pismum sativum* (Ritte et al. 1999) and in yeasts expressing monosaccharide carriers from lower and higher plants (reviewed by Büttner and Sauer 2000).

The H\(^+\)-dependent monosaccharide transporter of *O. europaea* cells exhibited broad specificity, being able to accept D-glucose, D-fructose, D-galactose, and D-xylose. Additionally, 2-deoxy-D-glucose and 3-O-MG are also substrates for the carrier. These results, together with those regarding kinetics and energetics, suggest that *O. europaea* monosaccharide carrier is a member of MST family, which comprises sugar transporters with a broad specificity, transporting a range of hexoses and pentoses with \( K_m \) values for the preferred substrate typically being 10–100 \( \mu \)M (Büttner and Sauer 2000). The data also showed that the affinity of the monosaccharide carrier for D-fructose was significantly lower than that for D-glucose, since 10 mM fructose inhibited only 20% the initial uptake rate of 0.2 mM D-[\(^{14}\)C]glucose. These results are in accordance with those obtained with cell suspensions of *D. carota* (Krook et al.
Proton-coupled sugar transporter activity can be regulated in two major ways: (i) indirectly, by regulating H+-ATPase activity, or (ii) more specifically by controlling the expression of sugar transporters at the transcriptional and post-transcriptional levels (Lalonde et al. 1999). It is believed that genes encoding monosaccharide transporters are differently regulated by sugars in higher plants. The analysis of expression of hexose transporter genes upon addition of glucose to photoautotrophic cell suspensions of Chenopodium rubrum, indicated that these genes are constitutively expressed and not regulated by sugar (Roitsch and Tanner 1994). These authors suggested that in higher plants the differential expression of sugar carriers does not depend on substrate induction, but rather reflects the existence of tissue-specific promoters not regulated by sugars. In contrast, the V. vinifera Vvht1 gene (hexose transporter 1) is up-regulated by sugar and plays an important role on sugar uptake into the flesh cells during the ripening of grape (Fillon et al. 1999). In this paper, evidence is provided indicating that in O. europaea cells alterations in sugar levels have a pronounced effect on monosaccharide transporter expression and/or activity, a carrier with high affinity being operational only when growth is carried out with low sugar supply. When growth was performed with 3% glucose, 3% sucrose, 3% mannitol or 3% galactose, glucose carrier activity was not detected, suggesting that its expression and/or activity are subject to sugar repression.

Three different pathways, a hexokinase-dependent system, a hexokinase-independent system involving an as yet unidentified glucose sensor or transporter, and a system dependent on active glycolysis downstream of hexokinase have been described for sugar sensing in plants (Rolland et al. 2001). The specificity of the repression of the monosaccharide/H+ symporter by different sugars suggests that a signal mediated by a plasma membrane sugar sensor/transporter with a broad specificity for monosaccharides is involved in the sugar-sensing mechanism, since the non-metabolizable analog 3-O-MG promoted the repression of the permease activity. However, the results do not exclude an additional involvement of a signal generated intracellularly.

**Material and Methods**

**Growth conditions**

Cell suspensions of O. europaea L. var. Galega Vulgar were maintained in 250-ml flasks on a rotatory shaker at 100 rpm, in the dark, at 25°C. Growth was in a modified Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with 0.0001% (w/v) nicotinic acid; 0.0001% (w/v) pyridoxine-HCl; 0.0001% (w/v) thiamine-HCl, 0.0001% (w/v) ascorbic acid, 4.6 µM kinetin, 4.5 µM 2,4-D; 25 mM MES buffer, 0.0005% (w/v) dithiothreitol and with the following carbon sources (w/v): 3% sucrose, 3% lactose, 0.5% or 3% glucose, 3% fructose, 3% galactose, 3% glycerol and 0.5% or 3% mannitol. Cells were subcultured every 12 d (6 d for 0.5% sugar-grown cells) by transferring 10-ml aliquots into 70 ml of fresh medium. Growth was monitored by determination of dry weight for estimation of maximum specific growth rates (μmax). Aliquots of 1–5 ml were filtered through pre-weighed GF/C filters (Whatman, Clifton, NJ, U.S.A.). The samples were washed with deionized water and weighted after 24 h at 80°C. Glucose consumption was monitored by the glucose oxidase method (Test-Combination, Boehringer Mannheim).

**Estimation of initial sugar uptake rates**

Cells were harvested after 6 d in culture, centrifuged, washed twice with ice-cold modified MS medium without sugar at pH 5.0, and resuspended in the same medium at a final concentration of 4 mg ml⁻¹ DW.

To estimate the initial uptake rates of α- or l-[^14]C]glucose, 1 ml of cell suspension was added to 10-ml flasks, under shaking (100 rpm). After 2 min of incubation, at 25°C, the reaction was started by the addition of 40 µl of an aqueous solution of radiolabeled sugar at the desired specific activity and concentration. The specific activities were defined according to the final concentration of the sugar in the reaction mixture, as follows: 500 dpn mmol⁻¹ (0.02–0.5 mM glucose), 100 dpn mmol⁻¹ (1–10 mM glucose) and 10 dpn mmol⁻¹ (10–50 mM glucose). Sampling times were 0, 60 and 180 s, time periods during which the uptake was linear. The reaction was stopped by dilution with 5 ml ice-cold modified MS medium without sugar, and the mixtures were immediately filtered through GF/C filters (Whatman, Clifton, NJ, U.S.A.). The filters were washed with 10 ml of the same medium and transferred to vials containing scintillation fluid (OptiPhase HiSafe II; LKB Scintillation Products). The radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation counter (Packard Instrument Co., Inc., Rockville, MD, U.S.A.). Results were corrected for non-specific binding of labeled sugars to the filters and/or the cells, by diluting the cells with 5 ml ice-cold modified MS medium without sugar, before the addition of labeled sugar. The values for the non-specific binding constant of labeled glucose, determined in a range of 0.02–50 mM sugar, were the following: for 3% glucose-grown cells, 0.060±0.003 µl min⁻¹ mg⁻¹ DW and for 0.5% glucose-grown cells, 0.049±0.002 µl min⁻¹ mg⁻¹ DW. These values are mean ± SD; n = 3. α-[U-[^14]C]glucose (305 mCi mmol⁻¹) and l-[U-[^14]C]glucose (55 mCi mmol⁻¹) were obtained from the Radiochemical Centre (Amersham).

**Determination of substrate specificity**

Inhibition of glucose transport by non-labeled sugars was assayed by adding simultaneously labeled and non-labeled substrate. The concentration range of labeled substrate varied from 0.02 to 0.2 mM and the final concentration of the cold substrate was at least 10-fold higher than the Kₐ value estimated for the carrier.

**Accumulation studies**

For accumulation studies, 10 ml of cell suspension was transferred to a 50 ml Erlenmeyer flask under shaking (100 rpm). After 2 min of incubation at 25°C, the reaction was started by the addition of an aqueous solution of radiolabeled 3-O-MG (specific activity = 3,000 dpn mmol⁻¹) at a final concentration of 0.1 mM. At appropriate times, 1-ml aliquots were taken from the reaction mixture into 5 ml ice-cold modified MS medium without sugar and filtered immediately through Whatman GF/C membranes. The filters were washed with 10 ml of the same medium, and the radioactivity was counted as indicated above. The intracellular concentrations of 3-O-MG were estimated as the ratio between the intracellular and the extracellular 3-O-MG concentration, using the intracellular volume obtained as indicated below. 3-O-methyl-l-[U-[^14]C]glucose (98 mCi mmol⁻¹) was obtained from the Radiochemical Centre (Amersham).
Estimation of initial rates of proton uptake

To estimate the initial rates of proton uptake upon addition of glucose, a standard pH meter (PHM 82 Radiometer A/S, Copenhagen, Denmark) connected to a recorder (Kipp and Zonen) was used as described earlier (Gerós et al. 2000) The pH electrode was immersed in a water-jacketed chamber with magnetic stirring. A total of 5 ml of O. europaea cell suspension in 10 mM potassium phosphate buffer (about 4 mg ml⁻¹ DW) were added to the chamber. The pH was adjusted to 5.0, and a baseline was obtained. The desired amount of glucose was added, and the subsequent alkalization curve was monitored. The slope of the initial part of the pH trace was used to calculate the initial rates of proton uptake. Calibration was performed with HCl.

Determination of intracellular volume

The methodology used to measure intracellular water volume was a modification of the methods previously described (Rottenberg 1979, De la Peña et al. 1981) and was based on the quantification of the relative distribution of two radioactive compounds in a cellular suspension: [³⁵C]methoxy-inulin to which biomembranes are impermeable and [²¹H]H₂O that equilibrates across biomembranes. After washing with modified MS medium without sugar, 2 ml of cell suspension were incubated with 5 μl of 250 mCi ml⁻¹ [²¹H]H₂O (Amersham; 5 Ci ml⁻¹), and 5 μl of 0.22 mg ml⁻¹ [³⁵C]methoxy-inulin (New England Nuclear; 5.2 mCi g⁻¹). The mixture was incubated for 30 min, and the cells were pelleted by centrifugation at 4,000 g, for 1 min. The supernatant (100 μl) was added to 5 ml of 1% (w/v) SDS and the same volume of SDS was added to the pellet. After overnight incubation, the mixtures were centrifuged, and the radioactivity of 40 μl of each supernatant was measured as described above. Intracellular water volume (V₃₅) was determined according to the expression:

\[ V_{\text{int}} = V_{\text{sup}} \left( \frac{\text{H}_{\text{pel}}}{\text{H}_{\text{sup}}} \right) - \left( \frac{\text{C}_{\text{pel}}}{\text{C}_{\text{sup}}} \right), \]

where \( V_{\text{sup}} \) corresponds to the volume of supernatant, \( \text{H}_{\text{pel}} \) and \( \text{H}_{\text{sup}} \) correspond to the \(^3\)H counts in the pellet and in the supernatant, respectively, and \( \text{C}_{\text{pel}} \) and \( \text{C}_{\text{sup}} \) correspond to the \(^1\)C counts in the pellet and in the supernatant, respectively. A value of 8±3 μl intracellular water mg⁻¹ DW (mean ± SE, \( n = 4 \)) was obtained.

Calculation of kinetic parameters

The data of the initial uptake rates of labeled glucose were analyzed by a computer-assisted non-linear regression analysis (GraphPad software, San Diego, CA, U.S.A.). By this method, the transport kinetics best fitting to the experimental initial uptake rates was determined, and then estimates for the kinetic parameters were obtained. Substrate uptake is presented as mean values ± SE and \( n \) denotes the number of independent experiments.

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References


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