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Solute uptake in plasma membrane vesicles from broad bean (*Vicia faba* L.) leaves

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SUMMARY

A number of physiological data that led to the conclusion of the existence of a proton/sucrose symporter mediating the uptake of sucrose into the phloem cells were obtained from broad bean (*Vicia faba* L.) The present paper investigates the possibility of studying the uptake of solutes at the plasma membrane level in this species in repsonse to an artificially imposed proton-motive-force. Plasma membrane vesicles were purified by aqueous two-phase partitioning from the microsomal fractions of broad bean leaves. The activity of selected marker enzymes showed that the plasma membrane vesicles were at least 90% pure. The imposition of an artificial proton-motive-force energized an active (against a concentration gradient) and transient uptake of both sucrose and valine. The maximum uptake rates were comparable for both sucrose and valine; sucrose was accumulated 6·2 times and valine 5·6 times above the diffusion equilibrium. The uptake of sucrose was saturable suggesting that the uptake was carrier-mediated. The affinity of the sucrose carrier for its substrate was 0·6 mM under energized conditions. These results confirm with a new material the possibility of studying proton-coupled transport of solutes directly at the membrane level, and are compared with data available from other species.

Key-words: amino acid uptake, broad bean, plant plasma membrane, sucrose uptake.

INTRODUCTION

In most plants, sucrose is the major photo-assimilate translocated in the phloem and is involved in energy transfer from autotrophic source organs to heterotrophic sink ones. The most commonly admitted explanation for the movement of assimilates in the phloem is the mass-flow model proposed by Münch (1930) and followers. According to this theory, the high concentration of sucrose in the phloem of leaves plays a major role in the translocation of assimilates. The very high concentration of sucrose in the phloem cells compared to the surrounding cells has been documented in different species (*Beta vulgaris*,

Abbreviations: DTT, dithiotreitol; SD, standard deviation.

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Geiger *et al.* 1973; *Amaranthus retroflexus*, Fisher & Evert 1982; *Populus deltoides*, Russin & Evert 1985). It has been proposed that the concentration of sucrose in the phloem cells is achieved by a carrier-mediated process catalysing H^+/sucrose co-transport from the apoplast (Giaquinta 1977; Delrot & Bonnemain 1978). This model emphasizes the importance of the transport of sucrose across the plasma membrane of mesophyll and phloem cells.

Despite some controversy addressing the possibility of symplastic loading in certain plants (Van Bel 1987; Turgeon & Gowan 1990; Turgeon & Beebe 1991), unequivocal evidence for apoplastic loading came from experiments run on leaves from two plants, sugar beet and broad bean (for review, see Giquinta 1983; Delrot 1989). The use of broad bean leaf discs, first chosen for the easy removal of the lower epidermis which allows for close contact between the incubation media and the apoplast, gave a lot of information concerning the characteristics of sucrose uptake. The kinetics of sucrose uptake indicated the existence of a high affinity sucrose transport system identified as a proton/sucrose symport mainly located in the phloem cells (Delrot 1981; Delrot & Bonnemain 1981). A similar system has also been proposed for the uptake of neutral amino acids (Despeghel & Delrot 1983).

Further work designed to identify the molecule involved in sucrose transport has been first conducted on the same material. An affinity labelling procedure with a thiol reagent (N-ethylmaleimide) was developed and led to the identification of a 42 kD polypeptide which could be involved in sucrose transport (Pichelin-Poitevin *et al.* 1987). This work was extended to sugar beet (Gallet *et al.* 1989), and functional evidence coming from immunological approaches (Gallet *et al.* 1991; Lemoine *et al.* 1989) and from reconstitution studies (Li *et al.* 1991a,b) confirmed the role of this polypeptide. The exact location of this polypeptide (mesophyll or phloem cells) remains to be determined.

In the last years, the study of solute uptake has turned to the design of very simple models such as plasma membrane vesicles in order to deal with true membrane transport without the problems linked to the use of tissue fragments (heterogeneity, diffusion) or even of protoplasts (metabolism, compartmentation). The first results showing that the uptake of sucrose could be studied in response to an artificially imposed proton-motive-force were obtained by our group (Lemoine & Delrot 1989) and others (Buckhout 1989; Bush 1989) on plasma membrane vesicles from sugar beet leaves. Further results concerning the uptake of amino acids were obtained on the same material (Gaillard *et al.* 1990; Li & Bush 1990) and this technique has recently been extended to other species (castor bean, Williams *et al.* 1990; spinach, Slone *et al.* 1991). Yet, in spite of the information gained from broad bean at the physiological level, this species has not been used for membrane transport studies in plasma membrane vesicles. The present work characterizes the uptake of sucrose into plasma membrane vesicles prepared from broad bean leaves.

MATERIALS AND METHODS

Preparation of plasma membrane vesicles

Broad bean (*Vicia faba* L. cv Aguadulce) was grown as described by M'Batchi *et al.* (1985). Fully expanded mature leaves from 3-week old plants were used. Plasma membrane vesicles were purified by two-phase partitioning from a microsomal fraction of leaf tissues according to Gallet *et al.* (1989) with minor changes. The final composition of the two-phase systems was 6·5% (w/w) dextran T 500, 6·5% (w/w) polyethylene glycol 3350, 300 mM sorbitol, 3 mM KCl, 5 mM potassium phosphate (pH 7·8) and 0·5 mM DTT. The

purified plasma membrane vesicles were pelleted for 45 min at 100 000 *g*, resuspended and equilibrated for 30 min in K-medium (50 mM K-phosphate pH 7·5, 300 mM sorbitol, 0·5 mM $CaCl_2$, 0·25 mM $MgCl_2$, 0·5 mM DTT). After pelleting for 45 min at 100 000 *g*, the vesicles were resuspended as a concentrated solution (about 10 mg protein ml^{-1}) in medium K without DTT, aliquoted, frozen in liquid nitrogen and stored at −80°C until further use.

Checking the purity of the plasma membrane vesicles

The purity of the plasma membrane vesicles was determined from the activity of selected marker enzymes. The activities of the tonoplast (nitrate sensitive), mitochondrial (azide sensitive) and plasma membrane ATPases (vanadate sensitive) were studied according to Hodges *et al.* (1972). The NADH-cytochrome c reductase activity was measured according to Hodges & Leonard (1974) and the glucan-synthase II activity was assayed as described by Ray (1977).

Sucrose uptake experiments

Uptake experiments were carried out according to Lemoine & Delrot (1989) except that DTT was omitted from the incubation media. Briefly, a 2 µl aliquot of vesicles (10 mg protein ml^{-1}) equilibrated for 30 min with K-medium containing 5 µM valinomycin was diluted in 400 µl of incubation media containing the radiolabelled substrate. At the end of the incubation, vesicles were collected by filtration and rinsed (Lemoine & Delrot 1989). For experiments in energized conditions, vesicles were diluted in Na medium buffered at pH 5·5 (same as K-medium except that it was buffered at pH 5·5 with 50 mM Na-phosphate and that it contained 5 µM valinomycin). Under these conditions, a ΔpH (alkaline inside) and an electrical gradient (ΔΨ, negative inside) resulting from the rapid exit of potassium ions from the interior of the vesicles in the presence of valinomycin were generated. For experiments in non-energized conditions (no gradient), the incubation medium was identical to the medium in which the vesicles had been resuspended (K-medium, pH 7·5). Depending on the experiments, the incubation medium also contained 1 mM [^{3}H]-sucrose or 1 mM [^{3}H]-valine (radioactivity: 64·75 kBq ml^{-1}). For the determination of the kinetic parameters of sucrose uptake, the sucrose concentration was varied between 10 µM and 3 mM.

Other methods

The metabolic fate of the sucrose taken up into the vesicles after 10 min of incubation was checked by paper chromatography of an ethanolic extract as described in Lemoine & Delrot (1989). Protein was measured according to Bearden (1978) using bovine serum albumin as a standard.

RESULTS

Purity of the plasma membrane vesicles

The different results presented in Table 1 give an indication of the degree of purity of the plasma membrane fraction obtained after two-phase partitioning. It is clear that only the specific activity of the plasma membrane ATPase (vanadate sensitive) increased in the plasma membrane fraction when compared with the microsomal fraction. This was confirmed by the extent of the inhibition exerted by vanadate on the ATPase activity at pH 6·5

Table 1. Comparison of the different marker-enzyme activities in the microsomal and in the plasma membrane fractions. Results are expressed as μmol Pi mg protein^{-1} h^{-1} for ATPases, μmol of reduced cyt c mg protein^{-1} h^{-1} for the NADH cyt c reductase and nmol of incorporated UDP-Glucose mg protein^{-1} h^{-1} for the glucan synthase II. The protein content was 21·3 ± 3·6 mg for the microsomal fraction and 1·04 ± 0·28 mg for the plasma membrane fraction. Results are the mean ± SD of 4–12 replicates. ND: not detected

	Specific activity	
	Microsomal fraction	Plasma membrane fraction
ATPase		
azide sensitive pH 9·0	0·28 ± 0·06	ND
nitrate sensitive pH 8·0	0·20 ± 0·19	0·09 ± 0·18
vanadate sensitive pH 6·5	0·53 ± 0·14	1·61 ± 0·21
NADH cyt c reductase	0·203 ± 0·01	0·085 ± 0·006
Glucan synthase II	9·16 ± 0·83	48·21 ± 7·12

(usually 70–80%). The only other enzyme displaying an increase (by a factor of 5·3) in specific activity is glucan synthase II, another marker of the plasma membrane. The increase in specific activity for glucan synthase II was higher than the increase noted for the vanadate-sensitive ATPase activity (5·3 vs 3·0). This could be explained by a more rapid inactivation of the glucan synthase II in the microsomal fraction due to the presence of phenolic compounds (Gallet *et al.* 1989). Transversely, for the other enzymatic systems investigated, an important decrease in specific activity was noted. Therefore, based on the ratio of the specific activity of the vanadate-sensitive ATPase to the total ATPases activities, it is possible to conclude that the plasma membrane vesicles were at least 94% pure. The determination of the ATPase latency in the presence of 0·02% Triton X-100 suggested that, in the fractions that had been frozen once for the purpose of storage, more than 70% of the purified plasma membrane vesicles retained their original (right-side-out) orientation (data not shown).

Sucrose uptake in plasma membrane vesicles

Due to the right-side-out orientation of the majority of the plasma membrane vesicles used in these experiments, it was not possible to energize the proton-sucrose co-transport by supplying ATP to the proton-pumping ATPase whose catalytic site lies on the cytoplasmic side of the membrane. Therefore, the uptake of sucrose was investigated in the presence of artificially imposed gradients (ΔpH and $\Delta\Psi$) as described by Lemoine & Delrot (1989). These two gradients represent the two components of the proton-motive-force and their orientation (alkaline and negative inside) is similar to the conditions found *in vivo*.

In the absence of gradient (non-energized conditions; Fig. 1, closed symbols), the vesicles took up very little sucrose (1·8 nmol mg protein^{-1} after 10 min of incubation). The internal volume of the vesicles as determined with $3H_2O$ and ^{14}C dextran, yielded a value of 2·3 μl mg protein^{-1} (Maurousset *et al.* 1992). Therefore, passive (or diffusive)

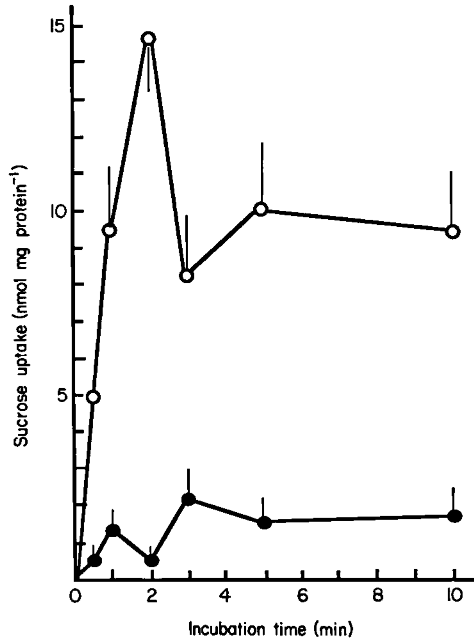

Fig. 1. Time-dependent uptake of 1 mM sucrose under energized conditions (ΔpH+$\Delta\Psi$, ○) and under non-energized conditions (no gradient, ●). The results are the mean ± SD of 18 replicates (five different experiments).

equilibration of sucrose into the vesicles from a 1 mM external solution should correspond to an uptake value of 2·3 nmol mg protein^{-1}. As the uptake value after a 10 min incubation under non-energized conditions was lower than the latter calculated value, it is reasonable to assume that, under these conditions, sucrose entered the vesicles by diffusion. On the other hand, in the presence of artificial gradients (ΔpH+$\Delta\Psi$), the values recorded for sucrose uptake (Fig. 1, open symbols) are much higher than in non-energized conditions. The maximal uptake value (14·6 nmol mg protein^{-1}) was obtained after a 2 min incubation and this corresponded to an accumulation ratio of 6·2 above the diffusion equilibrium. Sucrose was accumulated inside the vesicles against a concentration gradient, thus demonstrating that the uptake recorded was active.

The shape of the uptake curve (rapid accumulation of sucrose during the first 2 min then a decrease in the accumulation of sucrose) has been reported frequently in studies dealing with artificially energized uptake experiments. The reason for the decrease in the accumulation of the substrate lies in the transient nature of the artificial gradients created. The rapid decrease of both the electrical and pH gradients has been demonstrated recently on sugar beet plasma membrane vesicles (Lemoine *et al.* 1991). After the collapse of the energetical gradients, the substrate accumulated in the vesicles is effluxed down its concentration gradient.

The sucrose taken up by the vesicles after a 10 min incubation has been extracted with 80% ethanol and analysed by chromatography in order to test the possible involvement of sucrose metabolism in the uptake recorded. The radiolabelled substrate extracted from the vesicles co-migrated with sucrose and was cleaved by acid invertase. It is thus possible to conclude that sucrose was taken up and accumulated inside the vesicles without metabolism.

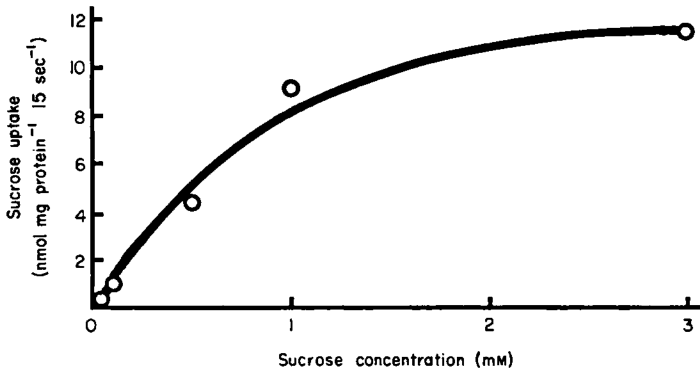

Fig. 2. Concentration dependent uptake of sucrose. The uptake values are expressed as the difference of uptake in energized conditions minus the uptake under non-energized conditions. The results are from one typical experiment (four replicates for each type of conditions).

Determination of the kinetic parameters of sucrose uptake

The experiments described in Figure 2 were run under energized conditions (ΔpH + $\Delta\Psi$) for an incubation time of 15 s, assumed to give a good estimation of the initial rate of uptake. In order to determine precisely the kinetic parameters, the uptake values recorded under energized conditions have been corrected for the corresponding uptake values under non-energized conditions at each sucrose concentration studied. The concentration dependence of sucrose uptake exhibited saturation kinetics consistent with carrier-mediated uptake. Graphic determinations from Figure 2 gave an apparent K_m value of 0·6 mM with a V_{max} of 12 nmol mg protein^{-1} 15 sec^{-1}. Yet double reciprocal plots (data not shown) showed a strong curvature occurring in the high sucrose concentration range, suggesting that the kinetics recorded are not of the regular Michaelis Menten type. Similar results have been reported and discussed with sugar beet plasma membrane vesicles (Lemoine & Delrot, 1989). The upward curvature of the double reciprocal plots may be due to the rapid dissipation of the proton-motive-force imposed when high sucrose concentrations are used, and to the lack of any mechanism re-establishing this force.

Valine uptake in plasma membrane vesicles

As noted in the introduction, sucrose is the major assimilate transported in the phloem. Nevertheless, it has been demonstrated by several authors that amino acids are also actively loaded in the phloem via a proton/sucrose co-transport (broad bean leaf-discs, Despeghel & Delrot 1983; *Commelina benghalensis*, Van Bel 1986). In order to get information on more than one substrate, we have investigated the uptake of a neutral amino acid, L-Valine. The possibility of studying the active uptake of valine with the gradients used to energize the uptake of sucrose has already been documented in sugar beet plasma membrane vesicles by Gaillard *et al.* (1990). The results presented in Figure 3 show that the uptake of 1 mM valine under energized conditions was also active and occurred against a concentration gradient. The maximal accumulation ratio was 5·6 between 2 and 5 min. Here again, as for sucrose and for the same reasons, the accumulation of valine was only transient.

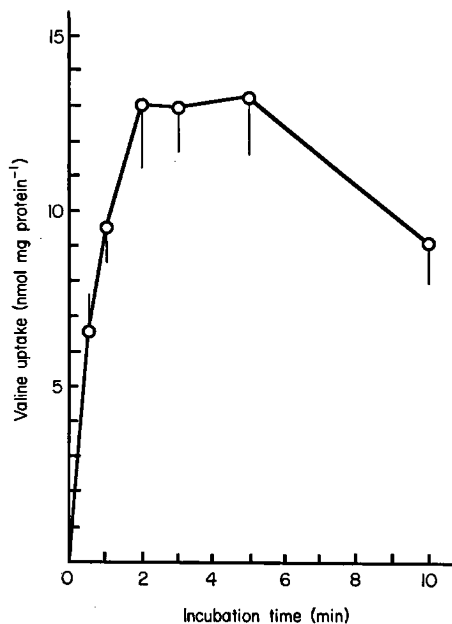

Fig. 3. Time dependent uptake of 1 mM valine under energized conditions ($\Delta pH + \Delta\Psi$). The results are the mean $\pm$ SD of 12 replicates (three different experiments).

DISCUSSION

The different results presented in this paper demonstrate the possibility of studying the uptake of sucrose and amino acids in plasma membrane vesicles purified from broad bean leaves. The use of two-phase partitioning allowed a very good purification of the plasma membrane vesicles (Table I) as noted previously on various materials. However the yield for this purification is lower than for sugar beet leaves (7 mg of plasma membrane protein starting from 125 g of leaves for sugar beet vs. 2–3 mg for broad bean).

The accumulation of sucrose and valine inside the vesicles in response to an artificial proton-motive-force is active because it occurs against a concentration gradient. The uptake of sucrose is saturable as demonstrated in Figure 2, even if the kinetics do not correspond to the Michaelis Menten type. The apparent K_m (0·6 mM) determined here is lower than the one noted for the so-called high-affinity uptake system described on leaf discs (2·6 mM, Delrot 1981). The lower value obtained with plasma membrane vesicles confirms the interest in studying the uptake directly at the plasma membrane level without the bias of the cell wall and/or metabolism of the substrate.

The use of plasma membrane vesicles to study the uptake of different substrates (mainly sugars and amino acids) has developed in the recent years. Therefore, it is very interesting to compare the results published so far. The results obtained in the present paper can easily be compared with those obtained with vesicles from sugar beet leaves in our lab because the same methodology was used. The maximum uptake rates under energized conditions are lower in vesicles from broad bean than in vesicles from sugar beet: 13·5 vs. 23 nmol mg protein^{-1} 2 min^{-1} (Lemoine *et al.* 1991) for sucrose and 12 vs. 40 nmol mg protein^{-1} 5 min^{-1} (Gaillard *et al.* 1990) for valine. This discrepancy can be explained by a difference in the efficiency of the energization. The values of the proton-motive-force

measured in plasma membrane vesicles was lower for broad bean (−170 mV, Maurousset *et al.* 1992) than for sugar beet (−200 mV, Lemoine *et al.* 1991). Assuming a 1/1 stoichiometry for the sucrose/proton symport, this difference in the proton-motive-force could energize a five-times-higher accumulation ratio in vesicles from sugar beet. However, in both cases the proton-motive-force was more than sufficient to account for the observed accumulation ratios (6·2 for sucrose in broad bean vesicles and 10 in sugar beet vesicles)

The apparent affinity for the substrate has only been determined for the sucrose carrier. In sugar beet a value of 0·3 mM was found (Lemoine & Delrot 1989) whereas in broad bean, the apparent K_m was 0·6 mM. Those K_m values for sucrose compare well with other values published so far: 0·45 mM (ΔpH, Buckhout 1989) and 1·2 mM (ΔpH, Bush 1989) in sugar beet vesicles, 0·87 mM in vesicles from *Ricinus* cotyledons (ΔpH + ΔΨ, Williams *et al.* 1990). The higher affinities obtained in our experiments can certainly be explained by a higher proton-motive-force created with our buffer system (higher buffer and potassium concentrations) compared to the buffer systems used by the other authors.

Another interesting point is the uptake of amino acids. In all systems tested so far, the uptake of amino acids is at least as important as the uptake of sucrose. This holds for sugar beet leaves (Lemoine & Delrot 1989; Gaillard *et al.* 1990; Bush 1989; Li & Bush 1990), *Ricinus* cotyledons (Williams *et al.* 1990) and for broad bean leaves (this paper). According to the major role attributed to sucrose in the mass-flow theory, one would expect plasma membrane vesicles of phloem origin to be able to accumulate sucrose at a higher ratio than amino acids. As pointed out by Buckhout (1989) and Lemoine *et al.* (1991), a significant proportion of the vesicles used in such experiments originate from mesophyll cells and only a limited amount should be of phloem origin. It is not possible to consider the present results as strictly representative of phloem loading. However one might speculate that the uptake of sucrose occurs at a high rate in plasma membrane vesicles coming from phloem cells whereas amino acids uptake occurs in vesicles coming from both phloem and mesophyll cells. This argument has no experimental support at the present time but we think that the question of the exact localization of the phenomena studied here will have to be addressed and answered.

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