Plant Physiol. (1979) 64, 61–64
0032-0889/79/64/0061/04/$00.50/0

Direct Evidence for a Sugar Transport Mechanism in Isolated Vacuoles[1]

Received for publication September 11, 1978 and in revised form February 9, 1979

MICHA GUY, LEONORA REINHOLD, AND DORIT MICHAELI
Department of Botany, The Hebrew University of Jerusalem, Israel

ABSTRACT

Sugar transport has been directly observed in isolated higher plant vacuoles for the first time. The latter were released from protoplasts isolated from the mesophyll of *Pisum sativum* L.

Uptake of L-glucose by the vacuoles was very slight in comparison with that of the D-glucose analog 3-*O*-methyl glucose (MeG), indicating, first, that a highly selective sugar uptake mechanism is seated in the tonoplast; and, second, that the mechanism was functioning in the isolated vacuoles.

MeG uptake was markedly sensitive to the pH of the medium, falling as the external pH rose. Addition of MgATP to buffered medium strongly promoted MeG uptake by vacuoles, but not by the protoplasts from which they were released. Treatment with the proton ionophore SF_{6847} drastically reduced uptake by the vacuoles, but had a lesser effect on uptake by the protoplasts. The inhibitory effect of SF_{6847} on uptake by the vacuoles was countered to a substantial degree by the addition of MgATP.

The influence of pH, the stimulatory effect of ATP, and the ATP-reversible inhibition by SF_{6847} all strengthen the conclusion that the observed sugar uptake reflected membrane function and was not due to a diffusional inward leak through damaged membranes.

The results are discussed in the light of currently held concepts regarding the driving force for sugar transport.

The question as to whether various membrane transport mechanisms are sited in the plasmalemma or the tonoplast has interested plant physiologists for a considerable period (*e.g.* 2). Evidence with regard to this siting in higher plant cells has until very recently been entirely indirect, based in large part on measurement of over-all fluxes across both plasmalemma and tonoplast. Lately, however, a start has been made in locating components of transport mechanisms at the membrane level. Membrane fractions rich in ATPase activity have been isolated (5). These membrane preparations have of necessity been of mixed origin, but it has been deduced that certain of the fractions consisted to a large extent of plasmalemma (5). The presence of an ATPase in a membrane fraction does not, of course, establish that it is a component of a transport mechanism, but it appears probable (5) that some of the ATPase activity detected may be related to ion transport.

Studies on isolated vacuoles have the obvious advantage that the membrane under observation is a single membrane of known origin. ATPase activity in intact isolated vacuoles, and in tonoplast vesicles prepared from them, has recently been reported by Lin *et al.* (8).

Direct evidence for the presence of uptake mechanisms in the tonoplast has so far not been adduced. Technical difficulties seem to have prevented uptake studies from being carried out on higher plant vacuoles in the past (12). We recently developed techniques for observing membrane transport in isolated protoplasts (4) and have now extended our studies to vacuoles released from these protoplasts. In the present communication we report studies on sugar uptake by isolated vacuoles, and bring direct evidence for the location of a selective sugar transport mechanism in the tonoplast.

MATERIALS AND METHODS

Pisum sativum L. var. Dan were grown in a growth chamber at 24 C and a photoperiod of 12 h. When the second leaf was almost fully expanded the terminal leaflet pair was excised for experiment. Details of the procedure for isolation of mesophyll protoplasts and of the composition of the suspension medium have already been reported (4).

Vacuoles were released from the protoplasts by the shearing force of ultracentrifugation through a step-density gradient (*cf.* 9). A high density protoplast suspension was layered on a two-step Ficoll gradient (Ficoll mol wt 400,000; 12 and 15% [w/v] in 0.6 M mannitol buffered at pH 7.7 with 20 mM Hepes) and centrifuged at 40,000 rpm in a SW 41 Beckman rotor for 2 h at 4 C. The majority of the vacuoles collected at the interface between the 12% Ficoll and the mannitol, although a certain percentage of the population formed a band between the 12 and 15% Ficoll. Usually only those at the former interface were used for the experiment. They were siphoned off, washed in buffered mannitol, and suspended in the suspension medium (4).

Uptake experiments were carried out by the techniques already described (4) except that the incubation medium was buffered at pH 5.4 with 40 mM Mes; vacuoles were retrieved from the incubation medium by centrifugation through the KCl-$CaCl_2$ mixture into a mixture of dibutyl and dioctyl phthallates, the composition of the mixture being such as to allow the entry of the vacuoles. The pellet was resuspended in water to bring about disintegration of the vacuoles, and aliquots were taken for assessment of accumulated label by scintillation counting. For internal standardization of the results other aliquots were taken for Lowry (10) determinations, so that variations in cell number as between samples could be corrected for. It was noted that Lowry-positive substances in addition to protein were present in vacuoles and protoplasts, but checks indicated that the *A* measured by this method was proportional to cell number. In certain experiments cell counts were made to allow calculation of the results on the basis of cell number.

SF_{6847} was kindly supplied by Professor M. Avron of the Weizmann Institute of Science.

RESULTS

Discrimination between Hexose Analogs. As in our previous study with isolated protoplasts (4) our first concern was to test the integrity of the vacuolar membranes and their capacity to function

[1] This research was supported by a grant from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel. The data are taken from a dissertation to be submitted by M. G. to the Hebrew University of Jerusalem in partial fulfillment of the requirements for a Ph.D. degree.

in transport. We therefore supplied the vacuoles with two hexose analogs, the unnatural sugar L-glucose, and MeG. The latter is a derivative of D-glucose which is known to be transported by the membrane mechanisms (16) but which is not metabolized. If uptake was essentially due to passive inward diffusion through membranes damaged in the course of isolation, the uptake curves for these two hexoses should have been closely similar. In fact strong discrimination between the two analogs was displayed by the vacuoles (Fig. 1). The difference in level of uptake became more pronounced with time, since the curve for MeG uptake continued to rise, whereas that for L-glucose uptake flattened.

Influence of pH. Sugar uptake was highly sensitive to the pH of the medium (Fig. 2). Uptake performance at pH 7.4 was much depressed as compared with that at pH 5.4. At pH 8.4 it was even further inhibited.

Effect of ATP. Addition of ATP to the external medium markedly enhanced MeG uptake by vacuoles (Fig. 3 and Table I). It is not likely that this effect was attributable to either chelation or a change in pH, since the ATP was brought to the pH of the buffered medium and then titrated with an equimolar amount of Mg ions (4).

For comparison, in some experiments a batch of the protoplasts from which the vacuoles had been liberated was tested at the same time as were the vacuoles. Here the effect of ATP, if any, was slight (Fig. 3c and Table I).

The enhancing effect of MgATP on uptake by vacuoles was evident both when the labeled sugar was diluted with unlabeled carrier to a final concentration of 10 mM, and in the absence of carrier (sugar concentration 22 μM). Even at the higher concentration the contribution to uptake of a passive penetration component, if any, would appear to be small, as indicated by the high specificity of uptake (Fig. 1).

Effect of Adding a Proton Inophore. It is currently thought that an electrochemical potential gradient for protons across the membrane may be an important driving force for sugar uptake. We therefore investigated the effect of SF_{6847}, the most potent of the acidic uncouplers (21), on sugar uptake, both in the presence and absence of added MgATP. Table I shows the results of two experiments, expressed on a "per cell" basis in order to allow direct comparison between the uptake performances of protoplasts and vacuoles, respectively.

ATP-treated vacuoles are seen to have about 44% of the uptake capacity of protoplasts. The discrepancy may be partly due to the absence of the cytoplasmic compartment, and also to damage suffered by some or all of the vacuoles during ultracentrifugation.

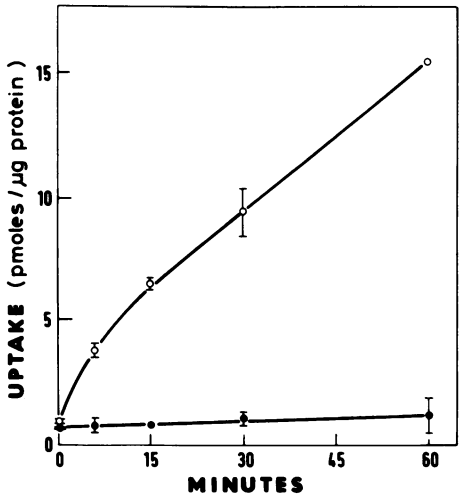

FIG. 1. Time course of uptake of MeG (○) and L-glucose (●) by vacuoles released from pea mesophyll protoplasts. External concentration of each sugar 10 mM. Error bars indicate ± 1 SE from plotted means.

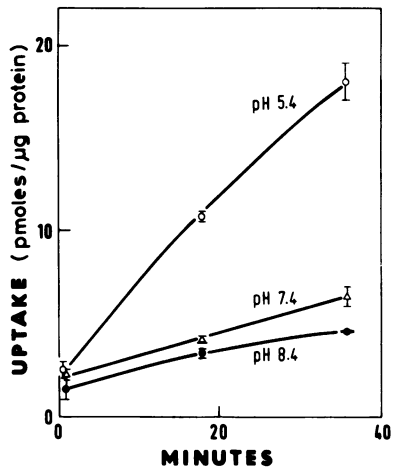

FIG. 2. Effect of pH on uptake of MeG by vacuoles released from pea mesophyll protoplasts. External MeG concentration: 7.5 mM. Buffer was a mixture of 40 mM Hepes and 40 mM Mes, titrated to required pH. Error bars indicate ± 1 SE from plotted means.

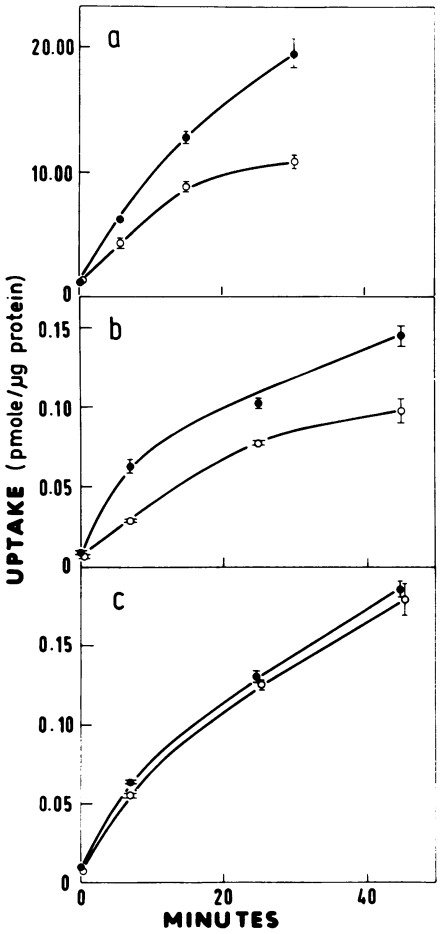

FIG. 3. Effect of addition of 3 mM MgATP to medium on uptake of MeG. a: Vacuoles; external MeG concentration was 10 mM. b: Vacuoles; external MeG concentration was 23 μM. c: Pea mesophyll protoplasts from which vacuoles used in (b) were released; external MeG concentration, 23 μM. MgATP was added 2 min before addition of labeled MeG. Error bars indicate ± 1 SE from plotted means. (○): Control; (●): +MgATP.

Furthermore, energization of the tonoplast *in situ* in the cytoplasm may be more efficient that that achieved by exogenous supply of ATP.

SF_{6847} markedly depressed MeG uptake by vacuoles (to 23% of

Table I. Effect of ATP and of the proton ionophore SF_{6847} on MeG uptake by pea mesophyll protoplasts and by vacuoles released from the protoplasts

The units are 10^5 x pmoles taken up per protoplast or vacuole in a 25 min period. The figures given are the means of triplicates ± SE. Mg-ATP concentration was 3 mM, and SF $_{6847}$ concentration 10^{-2} mM. MeG concentration was 22.7 µM and 10 mM in experiments 1, 2, respectively. The pH of the medium was 5.4.

Expt. no.	Additions to medium	Vacuoles	Protoplasts
1	---	3.51 ± 0.06	10.44 ± 0.45
	ATP	4.57 ± 0.09	10.55 ± 0.77
	SF_{6847}	0.80 ± 0.05	8.12 ± 0.61
	ATP + SF_{6847}	3.10 ± 0.08	9.90 ± 0.88
2	---	438 ± 27	
	ATP	743 ± 51	
	SF_{6847}	262 ± 14	
	ATP + SF_{6847}	402 ± 15	

the control at the lower sugar concentration). MgATP restored uptake to 88% of the control level (68% of the ATP-treated level). By contrast, SF_{6847} had only a slight effect on uptake by the protoplasts. It should be pointed out that owing to the fact that uptake in our experiments was very severely depressed at neutral and alkaline pH (Fig. 2) we were obliged to investigate the effect of SF_{6847} at pH 5.4 which is well below the optimal pH for its action on mitochondria (about 7.8 [21]). For this reason we were also obliged to use a relatively high concentration. Under our conditions SF_{6847} had no general toxic effect on the vacuoles leading to their disintegration. The number of cells recovered after incubation with SF, and their appearance under the microscope, did not differ from control samples.

DISCUSSION

The fact that the curve for uptake of L-glucose by vacuoles differed so strikingly from that for uptake of MeG (Fig. 1) is a clear indication, first, that the tonoplast is the seat of a discriminatory mechanism for the transport of sugars; and second, that the mechanism was functioning in these isolated vacuoles. The conclusion that the observed sugar uptake reflected a physiological function rather than mere passive inward diffusion through damaged membrane is strongly supported by several of our further findings. Uptake was highly sensitive to external pH (Fig. 2), and was markedly stimulated by the addition of buffered MgATP to the medium (Fig. 3 and Table I). The uncoupler SF_{6847} drastically inhibited uptake, an inhibition that could be countered to a considerable extent by the addition of MgATP (Table I).

The possibility might be raised that the uptake we followed was not so much into functioning vacuoles as into the protoplasts which "contaminated" the preparation to the extent of approximately 15 to 20% of the population. This possibility, however, can be unhesitatingly set aside. Comparison between vacuoles and the protoplasts from which they had been liberated revealed striking differences in uptake characteristics. MgATP affected uptake by protoplasts, if at all, to only a very slight extent; it strongly boosted uptake by vacuoles. Under the conditions of our experiments SF_{6847} inhibited uptake by protoplasts by about 20%, but depressed uptake by vacuoles more drastically (Table I). We concluded that the contaminating protoplasts contributed little to the uptake observed in the vacuolar preparation.

The lack of response of protoplasts to ATP, in contrast to the responsiveness of vacuoles, may be partly due to lack of penetration of externally supplied ATP to the tonoplast where an ATP-dependent mechanism connected with sugar uptake is apparently located. (The fact that uptake of α-aminoisobutyric acid by protoplasts was significantly stimulated by external ATP supply [4] may indicate that an amino acid transport mechanism accessible to ATP in the medium is located in the plasmalemma.) The availability of endogenous ATP generated inside the protoplasts would also reduce their responsiveness to exogenous ATP.

The question arises as to the source of energy for sugar transport in these isolated vacuoles in the absence of ATP in the medium. Perhaps no energization is required. Uptake as observed in these experiments was highly selective, but not "uphill," *i.e.* against the chemical potential gradient. The uptake system may be capable of bringing about facilitated diffusion in the absence of energy supply; in the presence of ATP it may become capable of active transport; for instance, the carrier might be converted to a non-transporting form at the inner membrane surface with concomitant hydrolysis of ATP (18). A second major possibility to be considered, and one which need not exclude the first, is that ATP primarily energizes an ion pump (*e.g.* an H^+ or K^+ pump), and that sugar uptake is a secondary consequence of the activity of this pump (19). We have in fact observed K-stimulated ATPase activity in the vacuoles. This ATPase activity increased with decrease in pH (Guy and Reinhold, in preparation).

The idea has lately been gaining ground among workers in the field that a "proton motive force" (13, 14) generated as the result of proton pumping could drive the transport of other solutes including sugars. For instance a membrane carrier might have a far higher affinity for sugars when in a protonated form, and net sugar flux would therefore depend on a higher proton concentration at one surface of the membrane than at the other (*cf.* 7). Recently a number of studies on plant cells have been published bringing evidence consistent with this view, and it has been suggested that sugars are taken up into cells by a proton-sugar co-transport system (*e.g.* 6, 7, 15). However, there is a major difference between the situation with regard to vacuoles and the model envisaged by the plant physiologists just cited. The latter have focused their thoughts on the plasmalemma: the negative plasmalemma potential of approximately −150 mv, together with the putatively high pH of the cytoplasm, provide a downhill electrochemical potential gradient for protons from the medium to the cytoplasm, the proton motive force. At the tonoplast, in striking contrast, the downhill electrochemical potential gradient for protons appears to be outward from the vacuole. The pH of the vacuole is generally held to be below that of the cytoplasm (20) and our preliminary observations on our isolated vacuoles are in agreement with this view and suggest a pH of less than 5.4 (that of our most acid medium). The tonoplast potential, according to the limited number of investigators who have measured it (20) is small and is positive; and again our preliminary observations on isolated vacuoles are in agreement. Thus, if proton motive force is driving sugar uptake into vacuoles the indications are that the mechanism would have to be an antiport system, and not a co-transport system.

The observation that sugar uptake by vacuoles was greatest when the external pH was low (Fig. 2) does not support the concept of proton antiport. However, it does not completely rule it out. The effect of external pH might reflect the optimal conditions for operation of a component of the system, as exemplified by the pH dependence of the tonoplast ATPase mentioned above. The depressive effect on uptake of the proton ionophore SF_{6847} when supplied in the absence of ATP can be regarded as evidence that a transmembrane proton gradient does in fact play a role in transport.

The relative lack of effect of SF_{6847} in the presence of ATP is among the observations that lead us to suggest that there are at least two modes of energy coupling for sugar transport in vacuoles. This suggestion is in line with current concepts of energy coupling for transport processes in bacteria. Transport may be either proton motive force-driven, or ATP-driven (3, 17) and within the same cell there may be several different systems, differently energized, for transport of the same solute (17). A transport system has also been reported where *both* proton motive force and ATP are required, and it has been suggested that this dual requirement represents energy fueling by ATP and regulation by the proton motive force (17).

In the present study the stimulatory effect of ATP was observable within the first few min, suggesting a direct action rather than an indirect one via the build-up of a proton motive force. As already pointed out, the uncoupler SF_{6847} had a much reduced effect in the presence of ATP. It is unlikely that ATP was generating a proton motive force faster than the uncoupler could dissipate it. It appears more likely that direct fueling of sugar uptake by ATP is possible in these cells. Direct energizing by ATP would also explain the relative lack of effect of SF_{6847} on uptake by protoplasts, if uncoupling was incomplete and native ATP was available in the protoplasts. A similar failure of uncouplers to abolish maltose transport in *Escherichia coli* was recently reported and regarded as evidence that proton motive force was not the sole driving force for transport (3).

The likelihood that sugar and amino acid transport systems in our cells can be directly energized by ATP is increased by the evidence (for references see 3) that in bacteria all of the sugar and amino transport systems which are sensitive to osmotic shock, *i.e.* which are dependent on "binding" protein which is removed by shock, use ATP directly. We have reported (ref. 1, and Reinhold in preparation) that sugar and amino acid uptake in leaf cells is indeed sensitive to osmotic shock.

Elucidation of the mechanism of sugar transport across the tonoplast presents a challenge all the more worth taking up in that the tonoplast, and not the plasmalemma, is the boundary membrane of the principal storage compartment for sugars in most plant cells (*cf.* 12). The present demonstration that tonoplast transport can be studied in isolated vacuoles, *i.e.* at membrane level, may open the way toward clarification of the molecular mechanisms and their energization.

Acknowlegement—We should like to thank Miss Michaela Rahat for excellent assistance.

LITERATURE CITED

1. Amar L, L Reinhold 1973 Loss of membrane transport ability in leaf cells and release of protein as a result of osmotic shock. Plant Physiol 51: 620–625
2. Epstein E 1976 Kinetics of ion transport and the carrier concept. *In* U Lüttge, MG Pittman, eds, Encycl Plant Physiol NS Vol 2B. Springer-Verlag, Berlin, pp 70–94
3. Ferenci T, W Boos, M Schwartz, S Szmelcman 1977 Energy-coupling of the transport system of *Escherichia coli* dependent on maltose-binding protein. Eur J. Biochem 75: 187–193
4. Guy M, L Reinhold, GG Laties 1978 Membrane transport of sugars and amino acids in isolated protoplasts. Plant Physiol 61: 593–596
5. Hodges TK 1976 ATPases associated with membranes of plant cells. *In* U Lüttge, MG Pittman, eds, Encylc Plant Physiol NS Vol 2A. Springer-Verlag, Berlin, pp 260–283
6. Hutchings VM 1978 Sucrose and proton co-transport in *Ricinus* cotyledons. Planta 138: 237–241
7. Komor E, Tanner W 1974 The hexose-proton co-transport system of *Chlorella*. J Gen Physiol 64: 568–581
8. Lin W, GJ Wagner, HW Siegelman, G Hind 1977 Membrane-bound ATPase of intact vacuoles and tonoplasts isolated from mature plant tissue. Biochim Biophys Acta 465: 110–117
9. Lorz H, CT Harms, I Potrykus 1976 Isolation of "vacuoplasts" from protoplasts of higher plants. Biochem Physiol Pflanz 169: 617–620
10. Lowry OH, NJ Rosebrough, AL Farr, RJ Randall 1951 Protein measurements with Folin phenol reagent. J Biol Chem 193: 265–275
11. MacRobbie EAC 1976 Electrolyte fluxes and energy coupling in plant cells. *In* R Passino, ed, Biological and Artificial Membranes and Desalination of Water. Pontificia Academia Scientiarum, Vatican, pp 375–402
12. Matile P, A Wiemken 1976 Interactions between cytoplasm and vacuole. *In* CR Stocking, U Heber, eds, Encycl Plant Physiol, NS Vol 3. Springer-Verlag, Berlin, pp 255–287
13. Mitchell P 1967 Translocation through natural membranes. Adv Enzymol 29: 33–87
14. Mitchell P 1977 Vectorial chemiosmotic processes. Annu Rev Biochem 46: 996–1005
15. Novacky A, CI Ullrich-Eberius, U Lüttge 1978 Membrane potential changes during transport of hexoses. Planta 138: 263–270
16. Reinhold L, Z Eshhar 1968 Transport of 3-*O*-methylglucose into and out of storage cells of *Daucus carota*. Plant Physiol 43: 1023–1030
17. Rhoads DB, W Epstein 1977 Energy coupling to net K^+ transport in *Escherichia coli* K-12. J Biol Chem 252: 1394–1401
18. Schafer B 1972 An examination of the energetic adequacy of the ion gradient hypothesis for nonelectrolyte transport. *In* E Heinz, ed, Na-linked Transport of Organic Solutes. Springer-Verlag, Berlin, pp 68–83
19. Slayman CL 1974 Proton pumping and generalised energetics of transport. *In* U Zimmermann, J Dainty, eds, Membrane Transport in Plants. Springer-Verlag, Berlin, pp 107–119
20. Smith FA, JA Raven 1976 H^+ transport and regulation of cell pH. *In* U Lüttge, MG Pittmann, eds, Encycl Plant Physiol, NS Vol 2A. Springer-Verlag, Berlin, pp 317–346
21. Terada H 1975 Some biochemical and physiochemical properties of the potent uncoupler SF_{6847} (3,5-di-*tert*-butyl-4-hydroxybenzylide-nemalononitrile). Biochim Biophys Acta 387: 519–532