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Membrane Transport of Sugars and Amino Acids in Isolated Protoplasts[1]

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ABSTRACT

A method has been developed for observing membrane transport in isolated protoplasts. Transport of sugars and amino acids has been studied in protoplasts isolated from the mesophyll of *Pisum sativum* L. That uptake was not due to passive diffusion through damaged membranes was demonstrated by supplying simultaneously two sugar stereoisomers, the one ^{3}H-labeled and the other ^{14}C-labeled. The protoplast membranes were sufficiently functional to discriminate strongly between these stereoisomers.

To characterize transport the nonmetabolized glucose analogue 3-O-methyl glucose (MeG) and amino acid analogue α-aminoisobutyric acid (AIB) were employed. When uptake was compared per unit of protein as between leaf strips and protoplasts prepared from the same tissue, it was estimated that the protoplasts had retained approximately 40 to 50% of the uptake ability of the whole cells. Uptake of neither MeG nor AIB by protoplasts was linear with time, but the tendency to flatten was more marked for AIB. Addition of Mg-ATP to buffered medium significantly promoted AIB uptake, an effect not ascribable to either chelation or pH. Transport of both MeG and AIB was markedly pH-dependent, uptake falling with rise in pH.

The stimulatory effect of Mg-ATP and the pH dependence confirm that uptake was not due to a diffusional inward "leak" but involved membrane function.

This work demonstrates the feasibility of using isolated protoplasts for membrane transport studies. The potential advantages of using protoplasts for such studies are pointed out.

The recent development of techniques for large scale release and isolation of protoplasts offers the opportunity of improving our understanding of membrane transport in plant cells. Interpretation of observed solute transport both into and out of the cell may be freed of the complicating factor of uptake into the Donnan space of the cell wall, or adsorption to wall constituents. Further, comparison of uptake properties of protoplasts and of vacuoles released from the protoplasts should lead to the identification of the membrane in which a particular transport mechanism is located. The question as to whether various transport mechanisms are located in the plasmalemma or the tonoplast is at present the subject of debate.

Successful experiments on transport in isolated protoplasts do not yet seem to have been reported. Taylor and Hall (13) have commented on the "poor ability" of the protoplasts in their investigation to accumulate ions. The problem involves not only the isolation of protoplasts in a condition such that their membranes are capable of transporting and retaining solutes, but also retrieval and analysis of protoplasts from the incubation medium without loss of the accumulated solute. In the present communication we report successful experiments investigating selective transport systems for sugars and amino acids in protoplasts isolated from the mesophyll of *Pisum sativum*.

MATERIALS AND METHODS

P. sativum L. var. Dan was grown in pots in the open air. Most of the experiments reported here were carried out in spring and early summer, when the photoperiod ranged from 12 to 14 hr, the mean day temperature from 16 to 25 C, and the mean night temperature from 11 to 19 C. When the second leaf was almost fully expanded the terminal leaflet pair was excised, surface-sterilized with 70% ethanol and cut into strips approximately 2 mm wide. The latter were incubated for 20 hr in 0.6 M mannitol (pH 5.8) containing 2% cellulysin (Calbiochem). The protoplasts were separated from cell debris by filtration through Miracloth and washed three times in the resuspension medium, each time being resedimented by centrifugation at 100*g*. The composition of the suspension medium was similar to that employed by Nagata and Takebe (8) except that KI, $CuSO_4$, the hormones, and the thiamine were omitted and the mannitol concentration was 0.6 M. Where a buffer was added, details are given in the text.

For uptake studies the labeled sugars and amino acids were added in appropriate concentration to the suspension medium. After the required uptake period the protoplasts were separated from the medium by rapid centrifugation through a 6-cm layer of a KCl-$CaCl_2$ mixture (0.25 M and 0.1 M, respectively) into a layer of dibutyl phthalate (*cf.* ref. 3, p. 327). Cell fragments and organelles did not enter this layer. The pellet was resuspended in water, bringing about disintegration of the protoplasts, and aliquots were then taken for assessment of accumulated label by scintillation counting, and of protein by the Lowry method (6).

Inspection under the microscope showed that the protoplasts released and used in these experiments were virtually all mesophyll.

As the results show, the *absolute* level for uptake rate tended to vary somewhat as between different experiments, carried out on different batches of protoplasts. Since the experiments were carried out over many months, weather conditions affecting the plants may be at least partly responsible for these differences in absolute values.

Radioisotopes were obtained from the Radiochemical Centre,

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Amersham. Samples were counted in a Packard Tri-Carb 3385 liquid scintillation spectrophotometer, quenching being corrected for by automatic external source ratio. The scintillation fluid contained toluene, Triton X-100, dimethyl POPOP, and PPO.

RESULTS AND DISCUSSION

Test of Ability of Membrane to Distinguish between Stereoisomers of Sugars. Our first concern was to test the possibility that the solute uptake we observed reflected, not so much the normal functioning of the membranes, as passive diffusion into the protoplasts through "leaky" membranes, *i.e.* through membranes which had become permeable owing to damage suffered in the course of isolation. We examined this possibility by supplying simultaneously, to the same protoplasts, D-glucose and its unnatural analogue L-glucose. The former was ^{3}H-labeled, and the latter ^{14}C-labeled. Figure 1a shows that the curve for uptake with time of the natural D-isomer lay far above that of the unnatural L-isomer. If uptake had been principally due to passive diffusion through damaged membranes, the curves for these two stereoisomers should have coincided. In additional experiments the uptake of the L-isomer was further compared with that of a derivative of D-glucose which is not metabolized, MeG.[2] Again, very different curves were obtained (Fig. 1b). We concluded that the membranes of the isolated protoplasts were in a sufficiently functional state to discriminate strongly between the natural and unnatural sugar stereoisomers.

Study of Uptake Mechanism for Sugars and for Amino Acids in Isolated Protoplasts. For more detailed study of membrane transport in protoplasts we used the amino acid analogue AIB and the glucose analogue MeG. These analogues are known to be transported by the membrane transport mechanisms in both animal and plant cells (9, 11) but they are not detectably metabolized. Transport processes can therefore be investigated in isolation from metabolic processes.

In the experiments reported here these two analogues have been supplied to the protoplasts simultaneously in the same medium (the sugar ^{3}H-labeled, and the amino acid ^{14}C-labeled). One of the advantages of this system is that any differences observed between the uptake behavior of the two substances are clearly not to be ascribed to biological variation between different aliquots of protoplasts. Preliminary experiments showed that the uptake curves for each substance, when supplied singly, did not differ from those for uptake from the combined medium. At the concentrations used, the sugar and the amino acid were not competing for uptake.

Figure 2 gives curves for uptake against time for the two substances. Uptake of MeG or AIB did not follow a simple linear form. The tendency to flatten with time is more marked for the amino acid than for the sugar. A similar diphasic uptake curve—a steeply rising phase followed by a less steeply rising phase—was observed for the uptake of another sugar analogue in *Ricinus* cotyledon tissue (4). One possible interpretation of these diphasic curves is that they represent uptake into at least two compartments, one filling up more rapidly than the other. An obvious possibility is that the rapidly filling compartment is the cytoplasm, and the "slow" compartment the vacuole. Experiments are in progress to examine this possibility directly by observations on isolated vacuoles as compared with protoplasts. A second possible explanation for the shape of the uptake curve is that it represents the approach toward equilibrium between influx and efflux.

Uptake Performance of Protoplasts as Compared with Whole Tissue. When cell walls were removed from bacterial cells by treatment with lysozyme and EDTA, the resulting spheroplasts were reported to retain approximately 40% of the amino acid uptake ability of the whole cells (2). In more recent studies (Halpern, personal communication) this percentage has been raised to about 70. A comparison of this kind is more difficult to achieve in the case of higher plant tissue. One of the chief complicating factors is the fact that a substantial proportion of the solute taken up by strips of leaf tissue is contained, not within differentially permeable membranes, but extracellularly, in the free space of the tissue. In our previous studies of amino acid uptake by leaf tissue (1, 10, 11) the dimensions of the leaf strips

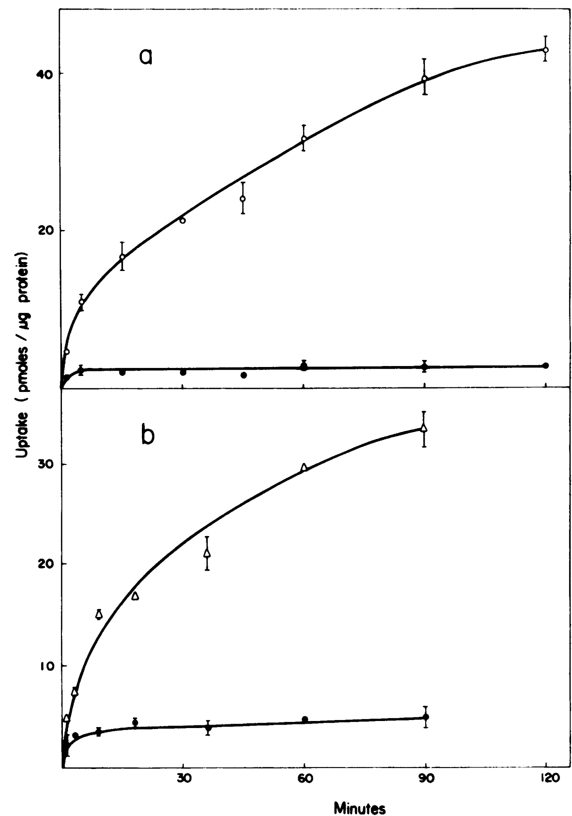

FIG. 1. Time course of uptake of D-glucose (○), L-glucose (●), and 3-O-methyl glucose (△) by isolated pea mesophyll protoplasts. Error bars indicate ± 1 SE from plotted means. The bars have been omitted when their size is less than the symbol shown. a: ^{3}H-labeled D-glucose and ^{14}C-labeled L-glucose supplied simultaneously, both at 10 mM. b: ^{3}H-labeled 3-O-methyl glucose and ^{14}C-labeled L-glucose supplied simultaneously, both at 10 mM.

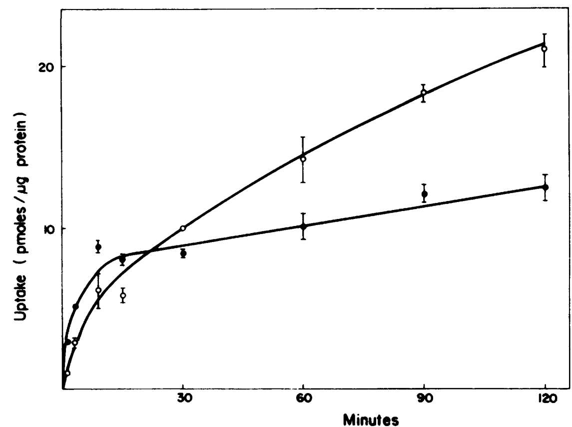

FIG. 2. Uptake of MeG (○) and AIB (●) by isolated pea mesophyll protoplasts. ^{3}H-labeled MEG and ^{14}C-labeled AIB were supplied simultaneously, both at 10 mM. Error bars indicate ± 1 SE from plotted means. The bars have been omitted when their size is less than the symbol shown.

[2] Abbreviations: AIB: α-aminoisobutyric acid; MeG: 3-O-methyl glucose.

were chosen such that membrane transport into the cells would not be limited by the rate of diffusion through the tissue from the external medium to the transport sites (11). These earlier studies indicated that washing for 15 min was an appropriate period for removing AIB from the free space of leaf strips of these dimensions. Clearly, it must be borne in mind that the estimate of the absolute amount of solute taken up in a specified time is open to error. Too long a wash may result in appreciable loss of solute from within the cells themselves, while too short a wash will leave appreciable amounts of solute in the free space.

In the present investigation we have compared uptake/unit of protein as between leaf strips 0.8 mm wide and protoplasts prepared from the same tissue. Aging promotes the uptake capacity of leaf strips (1, 11) and the strips were therefore aged under conditions parallel to those prevailing during release of the protoplasts, *i.e.* immersion for 20 hr in 0.6 M mannitol. For measurement of MeG and AIB uptake the strips were incubated in a medium of the same composition as that in which the protoplasts were suspended. At the end of the uptake period the leaf strips were subjected to the standard 15-min wash. MeG taken up by the protoplasts from a 10 mM solution was approximately 40 to 50% of that taken up by the leaf strips as estimated under these conditions. A typical experiment is shown in Table I. In the case of AIB, uptake by protoplasts was also about 45% for short incubation periods, but the percentage tended to fall as the duration of incubation rose. This followed from the fact that the progress curve for uptake of AIB by leaf strips continued to rise with time (see also refs. 1 and 11) rather than tending to flatten as in the case of isolated protoplasts.

A further factor to be taken into account when considering the uptake performance of isolated protoplasts as compared with that of leaf tissue is that the latter comprises a heterogeneous assembly of cell types, some of which, *e.g.* phloem, may have higher uptake capacities. Our suspensions of protoplasts, on the other hand, contained virtually only mesophyll cells.

Effect of Adding ATP to the Medium. The addition of ATP to the external medium boosted uptake of both the sugar and the amino acid. There have been a number of reports that exogenously supplied ATP can stimulate various transport processes in plant tissues, but doubts have been raised as to whether these effects have been specific (related to energy supply), or indirect. It has been suggested that the effects may be due to chelation of divalent cations by ATP (7) or to a change in pH (14). We have, therefore, taken the following precautions. The ATP was brought to the pH of the medium and then titrated with an equimolar amount of Mg ions. The Mg-ATP formed will have lacked chelating ability. It was then added to a medium buffered with 40 mM MES. Figure 3 shows that even under these conditions ATP stimulated the uptake of AIB. In the earlier experiments, where the pH was less rigorously controlled and where ATP, and not Mg-ATP, was supplied the effect was larger (approximately 50% in the case of MeG and 100% in that of AIB). This suggests that chelation

Table I. Uptake of MeG and of AIB by pea leaf strips as compared with that by protoplasts prepared from the same tissue.

The units are pmoles per µg protein. The figures given are the means of duplicates. ^{3}H-labelled MeG and ^{14}C-labelled AIB were supplied simultaneously, both at 10 mM. Width of leaf strips 0.8 mm.

Substance supplied	Uptake period (min)	Uptake by leaf strips (a)	Uptake by protoplasts (b)	b/a
MeG	5	5.6	2.9	0.52
	10	9.3	4.8	0.52
	15	13.8	7.0	0.51
	25	20.4	10.1	0.49
	45	30.3	14.3	0.47
	60	37.3	16.2	0.43
AIB	5	4.3	1.94	0.45
	10	7.9	3.6	0.45
	15	12.3	5.4	0.44
	25	15.6	6.6	0.42
	45	20.7	8.3	0.40
	60	23.7	9.1	0.38

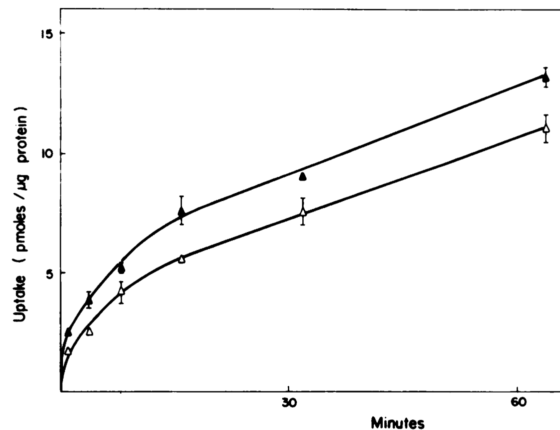

FIG. 3. Effect of addition of 3 mM Mg-ATP to the medium on uptake of AIB by isolated pea mesophyll protoplasts. (▲): +Mg-ATP, (△): −Mg-ATP. The medium was buffered at pH 5.4 with 40 mM MES. External AIB concentration 10 mM. The Mg-ATP was added to the protoplasts 10 min before addition of AIB. Error bars indicate ± 1 SE from plotted means. The bars have been omitted when their size is less than the symbol shown.

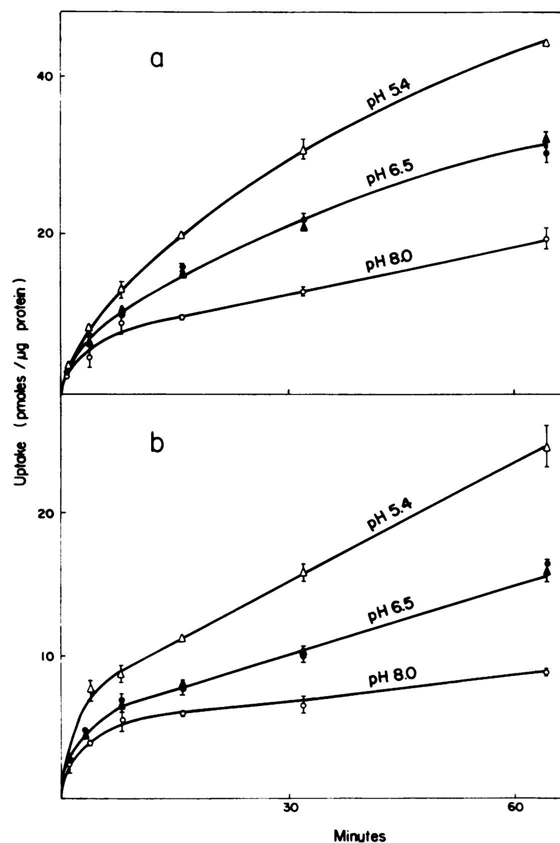

FIG. 4. Effect of pH on the uptake of MeG (a) and AIB (b) by isolated pea mesophyll protoplasts. (△, ▲): MES buffer, 40 mM; (○, ●): HEPES buffer, 40 mM. External concentration of MeG and AIB both 10 mM. Error bars indicate ± 1 SE from plotted means. The bars have been omitted when their size is less than the symbol shown.

and/or pH may in fact have contributed to the ATP effect observed under these conditions. However, the statistically significant effect shown in Figure 3 ($P < 0.001$ as indicated by a t test) indicates an action of ATP on AIB uptake that cannot be ascribed to chelation or pH. The effect of ATP is most marked during the initial stages of uptake. A possible explanation is that the progress curve for uptake does in fact represent the sequential filling up of

an outer and inner compartment; the effect of externally supplied ATP is confined to the membrane bounding the outer compartment. This stimulation of uptake by Mg-ATP during the initial stages provides additional important evidence that the uptake observed in our protoplast preparations is not a simple diffusional "leak" inward but involves the physiological functioning of the membrane.

Effect of pH. When the medium was buffered at various pH values, uptake, not only of the amino acid but also of the sugar, was seen to be strongly pH-dependent. Since MES buffer was used at low pH values and HEPES at high pH values, we have checked that the difference in buffer ion was not at least partly responsible for the pH effect. Both buffers can be used at pH 6.5, and Figure 4 shows the time courses for uptake at this pH in each of the buffers, as well as at pH 5.4 (MES buffer) and at pH 8 (HEPES buffer). There is no significant difference between the points for the buffers at pH 6.5. Figure 4 shows a clear fall in uptake with rise in pH both for MeG and for AIB, again confirming that uptake is not due to passive diffusion.

This pronounced effect of H^+ ion concentration in the external solution is of special interest in view of the possibility (*e.g.* 12) that the transport of sugars and amino acids may be dependent on the simultaneous co-transport of protons. In current experiments we are investigating this possibility. Komor and Tanner (5), who have brought evidence for such co-transport in *Chlorella*, reported a pH optimum of 5.4 for the transport of 6-deoxyglucose.

This work has demonstrated the feasibility of carrying out membrane transport studies on isolated protoplasts. Apart from the advantages already pointed out in the introductory section, removal of the diffusion resistance and "unstirred layer" constituted by the wall also opens the way to more precise kinetic studies than were possible in the past.

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