Protonation and Light Synergistically Convert Plasmalemma Sugar Carrier System in Mesophyll Protoplasts to its Fully Activated Form

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ABSTRACT

The course of sugar fluxes into and out of protoplasts isolated from the mesophyll of Pisum sativum L. has been followed over brief time intervals (minutes). Light strongly stimulated net sugar influx at pH 8 as well as at pH 5.5. The proton conductor carbonyl cyanide m-chlorophenylhydrazone (CCCP) inhibited initial influx in the light, both at pH 8.0 and at pH 5.5. CCCP was without effect in the dark at either pH. All these results applied both to sucrose and to the nonmetabolizable glucose analog 3-O-methyl-D-glucose.

When protoplasts at pH 5.5 were transferred from light to darkness, "stored" light driving force maintained uptake in the dark at the full light rate for the first 7 minutes. At pH 8, however, even 4 minutes after transfer to dark, uptake was well below the light rate. Initial uptake rates over a range of external concentrations were derived from progress curves obtained in the light and in the dark, both at pH 5.5 and at 7.7. When initial rate was plotted against concentration, simple Michaelis-Menten kinetics were observed only under the condition pH 5.5, light. In the dark at both pH values, and in the light at pH 7.7, complex curves with intermediate plateaus were obtained, strongly resembling curves reported for systems where mixed negative and positive cooperativity is operating.

The same "Km for protons" was observed in the dark and in the light (10⁻² molar). Switching protoplasts in the dark from pH 8 to 5.5 failed to drive sugar transport by imposed protonmotive force, as judged by lack of sensitivity to CCCP. Switching protoplasts which had been driven in the dark to pH 5.5 to pH 7 induced net efflux of sugar. Flux analysis showed that this effect was entirely due to the prompt fall in influx.

It is concluded from the kinetic experiments that protonation alone is not sufficient to convert the sugar transport system to its fully activated high affinity form. A further light-dependent factor which acts synergistically with protonation is required.

The boundary membrane of a naked protoplast is in direct contact with the solutes in the external medium, without the intervening compartments of the cell wall and intercellular spaces which are present in whole organs or tissue slices. These external compartments have complicated flux measurements in higher plant cells in the past, because initial flux should be estimated from the intracellular content after brief influx periods; but most of the substance taken up into a tissue after short-term incubation is present in the extracellular compartments (2).

Use of protoplasts isolated from pea mesophyll cells recently enabled us to measure initial flux of sugars through the plasmalemma in darkness and in light, and to detect within very few minutes changes in flux which occurred as a result of transition from light to darkness (8, 14). We reported that light uptake differed from dark uptake in that it was sensitive to the proton conductor CCCP² but initially stable to various ATPase inhibitors and to arsenate. Dark uptake from the start was sensitive to these latter inhibitors but insensitive to CCCP. On transition from light to dark at pH 5.5, the light driving force was apparently "stored" for the first 7 min in the dark. During this period not only was uptake at the accelerated light rate but it showed the inhibitor sensitivity characteristic of light and not of dark conditions.

In the present communication, we report further on the characteristics of light and dark uptake in protoplasts, particularly in relation to pH. It is currently widely believed (1, 3) that membrane sugar transport involves co-transport of sugars and protons, driven by the protonmotive force (the electrochemical potential gradient for protons). According to a generally accepted model (e.g. 9), protonation of a transport protein converts the sugar transport system to a form with a high affinity for sugar, capable of uptake from media of low sugar concentration. Among the findings we present below is a demonstration, based on studies of initial velocity versus concentration, that in our plasmalemma system protonation alone is not a sufficient condition for conversion of the system to its fully activated high affinity form. A further light-dependent factor is required, which acts synergistically with protonation.

MATERIALS AND METHODS

The plant material was Pisum sativum L. var. Dan. The methods for growing and sampling the material, as well as for isolation of protoplasts, were as described earlier (6, 8). Cellulysin concentration was 1%. In some experiments, the duration of Cellulysin treatment was shortened from 20 h at room temperature to 2 h at 30 C; no effect of change in duration of Cellulysin treatment could be detected on rate or kinetics of uptake.

Pretreatment and incubation took place in the modified Nagata medium (6) buffered with Mes + Heps (each 50 mm). Mannitol concentration was 0.5 M. Retrieval of protoplasts from the incubation medium was carried out as described in (7) as was the subsequent procedure for assaying radioactivity and internal standardization.

The light source for light treatments was as given in reference 8. Protoplasts to be incubated in the light were also pretreated for

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2 Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; MeG, 3-O-methyl-D-glucose.
RESULTS

Sensitivity to Light and to CCCP at Different External Proton Concentrations. Figure 1a gives progress curves for MeG uptake, in the light and the dark, at two pH values, during the "plasmalemma uptake phase" (8). It shows that the effect of light on MeG uptake, previously reported when the pH of the medium was 5.5, is also very marked at pH 8.

In addition Figure 1a shows the effect of the proton conductor CCCP on MeG uptake in the light at the two pH values. CCCP reduces uptake in the light to the dark level. The interesting fact emerges that the sensitivity of uptake to pH remains undiminished in the presence of the proton conductor, although presumably the latter collapses the protonmotive force, either partly or completely.

The initial slope for uptake at pH 5.5 is about 3 times that at pH 8, both in the presence and absence of CCCP.

This result may also be expressed from a different view point—the magnitude of the inhibitory effect of CCCP is no less at pH 5.5 than at pH 8, although in the latter case ΔpH across the membrane has presumably been abolished or reversed.

CCCP was without appreciable effect on uptake in the dark, either at pH 5.5 (8) or at pH 8.0 (not shown).

Sensitivity of Sucrose Uptake to Light, to pH, and to CCCP. Sucrose is the "translocation sugar," the principal form in which photosynthate is exported by mesophyll cells, and may pass in and out of mesophyll cells en route to the vein. It was of interest to check whether the membrane transport characteristics we observed for glucose and other sugars (8) also applied to sucrose. Figure 1b demonstrates that sucrose uptake closely resembles MeG uptake in its sensitivity to light and to pH. It is also sensitive to the proton conductor in the light but not in the dark (not shown).

Effect of Light to Dark Transition at Acid and Basic pH Values. If protoplasts in a medium at pH 5.5 are pretreated for 1 h in the light, subsequent sugar uptake in the dark is at the accelerated "light" rate for the first 7 min (8). Figure 2 shows an example of an experiment to check whether this effect of "stored driving force" is also evident at pH 8 (i.e. in the absence of a positive Δ[H+] between medium and cytoplasm). At pH 5.5, uptake for the first 7 min in the dark after light pretreatment again followed the exact course of light uptake (Fig. 2). At pH 8.0, even 4 min after transfer from light to dark uptake was well below the light rate. Nevertheless the rate of uptake by the light pretreated protoplasts was somewhat above the dark rate.

Kinetics of Uptake (V versus S) in Dark and in Light at Two pH Values. Figure 3a shows initial rate of uptake as a function of external MeG concentration at pH 5.5 in the light and in the dark. Figure 3b shows the same relationship for pH 7.7, again in the light and in the dark. Each point in these figures has been obtained from a time course of uptake at each concentration. Uptake was

at least 1 h in the light; "dark" samples spent this period in darkness.

assessed after 0.5, 1.5, 3.5, 6, and 9 min. The initial slope was derived from the progress curves and is given in Figure 3. During the 9 min, uptake was observed to be not far from linear.

Figure 3, a and b, shows that a curve close to a rectangular hyperbola was obtained only under one set of conditions—pH 5.5, in the light. This curve approximates to classic Michaelis-Menten kinetics, as may be seen in the inset, where the plot V/S versus V gives a reasonable straight line. Under all of the other conditions shown in Figure 3, a and b, the curves obtained are not rectangular hyperbolas; they are curves with intermediate plateaus, strongly suggesting that cooperative effects are involved. They resemble curves obtained for systems where mixed negative and positive cooperativity is operating (10; see under “Discussion”).

The “Km for Protons” in the Dark as Compared with the Light. Dark uptake was earlier shown to be very different from light uptake in sensitivity to inhibitors (8). The question arises as to whether a totally different system mediates uptake in the dark, or whether the same carrier system operates under both conditions but is energized differently in light and in dark.

As an approach to this question we have studied the affinity of the system for protons in the light and in the dark. Time curves for uptake were obtained over the pH range 5 to 8 at intervals of 0.5 pH units. The initial rates of uptake were derived from these time curves and are shown as a function of hydrogen ion concentration in Figure 4. Both curves approximate to rectangular hyperbolas. If a protonated carrier complex in fact plays a central role in sugar transport, then the results shown in Figure 4 may allow assessment of the Km of the carrier for protons (cf. 5, 9).

Linear transformation of the hyperbolas (see inset) indicate about the same Km—10⁻⁷ M, or pH 7, in both cases. (The slope of the lines give 1/Km.) The intercept on the V axis (i.e. Vmax, rate of uptake at saturating proton concentration) is, however, more than 4-fold higher in the light than in the dark.

Results obtained when the external MeG concentration was 10 mM, instead of 1 mM as in Figure 4, show a very similar pH dependence (6, and unpublished data).

Attempt to Drive Sugar Uptake by Imposed pH Gradient in the Dark. Our results are consistent with the notion (see under “Discussion”) that in the light uptake may be driven by protonotive force. The CCCP stability of dark uptake, on the other hand, suggests that this force is not operating in the dark. A possible explanation might be that in the dark ΔpH across the membrane is not maintained, and that ΔpH is the most influential component of the protonotive force at pH 5.5. We have attempted to impose an artificial proton gradient across the plasmalemma in the dark by first incubating protoplasts at relatively high pH (pH 8) then switching them to low pH (pH 5.5). As criterion for the operation of protonotive force we have used sensitivity of sugar uptake to the proton conductor CCCP. This technique has revealed protonotive force driven sugar uptake by a similarly imposed ΔpH in E. coli (4). In our case, measurements made even only 3 min after the switch from pH 8 to pH 5.5 failed to detect CCCP sensitivity of MeG uptake in the dark. Moreover, the reverse switch (incubation with MeG at pH 8 after pretreatment at pH 5) failed to reveal a slower initial rate of uptake, as compared with protoplasts pretreated at 8. A lower initial rate might have been expected as a result of the imposed outwardly directed ΔpH. In all cases, the rate of sugar uptake reflected the pH of the medium at time of sugar supply, in accordance with Figure 4.

This last fact also emerges from the experiment shown in Figure 5 to be discussed below.

Effect on the Unidirectional Fluxes of Switching Protoplasts from pH 5.5 to 7.0. In this experiment protoplasts were allowed to take up [¹⁴C]MeG for 50 min either at pH 5.5 or at pH 7.0. They were then centrifuged briefly and resuspended in MeG medium at the same concentration but now labeled with ³H. Aliquots of protoplasts were removed at intervals and assayed for both ³H and ¹⁴C, allowing assessment of unidirectional influx and efflux, respectively. Half the protoplasts which had been at pH 5.5 were switched to pH 7 at the time of transfer to ³H-labeled medium, the other half continued at pH 5.5. The experiment was carried out in the dark.

The following points emerge clearly from Figure 5 which presents the results:

1. Net influx was from the very start of the uptake period lower at the higher pH.
2. Switching of protoplasts from pH 5.5 to 7.0 induced net efflux of sugar. Sugar was thus lost from the protoplasts, even though the bathing medium was at the same sugar concentration as the previous one. This result strongly suggests that, even under these dark conditions, an internal sugar concentration above the external had been built up in some compartment of the cell during incubation at pH 5.5.
3. The net efflux of sugar after transfer to pH 7.0 was entirely due to the prompt response of influx, which fell quickly from the level which had characterized uptake at pH 5.5 to that which characterized uptake at pH 7.0. A net loss of sugar after a similar pH switch has been observed for algae, and has been attributed to enhanced efflux (9), although in the latter case no flux analysis was performed.

4. Unidirectional efflux was affected by the pH switch to only a slight degree. This result suggests that unidirectional flux is determined mainly by the H⁺ concentration on the same side (the "cis" side) of the membrane. The "trans" H⁺ concentration (i.e. the concentration on the opposite side) had only a small effect on efflux, but it was consistent in repeated experiments—efflux was somewhat depressed by the switch to lower trans H⁺ concentration.

DISCUSSION

Evidence we have collected here and in an earlier communication (8) would be consistent with the current hypothesis that the driving force for membrane sugar transport is the electrochemical potential gradient for protons (ΔµH⁺), but only in the light. In particular, the "stored" driving force evident during the first few minutes after transition of protoplasts from light to darkness at pH 5.5 is most reasonably explained as residual ΔµH⁺. This follows from a consideration of its stability or sensitivity respectively to a variety of inhibitors (8). The question arises from the present study as to why the stored driving force is capable of maintaining initial dark uptake at the full light rate if the protoplasts are at pH 5.5, which this is not the case at pH 8.0. We offer the following possible solution to this question:

At pH 5.5, the electric potential component of ΔµH⁺, Δφ, is much less influential on sugar uptake than is ΔpH. This has been observed for Chlorella by Schwab and Komor (16); and has also been reported by Kacab and his collaborators (see 12) for E. coli membrane vesicles with regard to a considerable number of transport systems including that of glucose-6-P. At pH 8, on the other hand, in the absence of an inwardly directed ΔpH, uptake is sensitive to Δφ. This has been observed both for Chlorella (16) and for the E. coli system (12). Light may greatly stimulate the plasmalemma-sited proton extrusion pump. This has been shown for Nitella by Spanswick (17, 18) and for oat protoplasts by Rubinstein and Tattar (15); and has also been proposed by van Bel (21) for tomato internode discs. (In our system, the pump may scarcely operate in the dark, as judged from the observation that fuscosin only stimulates sugar uptake in the light [Guy et al., in preparation].) On transfer to darkness, loss of electrogenic pump activity may result in a drop of potential (noted by Spanswick, 17), the half time for decay being about 0.5 min (23, p. 246). The driving force for sugar uptake at pH 8 may thus have diminished too fast in our protoplasts on transfer to darkness for us to observe it. We deduce that the ΔpH created by the pump, which is the principal driving force at pH 5.5, decays far more slowly and thus allows uptake at the accelerated light rate for some minutes after transition to dark.

A striking finding in the present investigation is that classic Michaelis-Menten kinetics are observed when uptake is taking place at pH 5.5 in the light, but that in the dark, or at high pH in both light and dark, the curves relating initial uptake velocity to external MeG concentration are multiphasic and show intermediary plateaus (Fig. 3), suggesting complex cooperativity (see below). It has been suggested for Chlorella (9, 19) as for many systems that the glucose carrier exists in two different states for binding sugar, a protonated form with a high affinity for sugar, and an unprotonated low affinity form. Between approximately pH 6.5 and 8.5, in the case of Chlorella, it is claimed that a mixture of the two forms is present and that total uptake is the result of the joint operation of both forms of carrier. A disadvantage of this proposal, however, is that curves with bumps (cf. 19) cannot result from the sum of two Michaelis functions. Summed Michaelis functions always give a smooth curve (e.g. 13). Curves with bumps or intermediary plateaus are, however, known for a number of enzymes (10, 20), and some of them bear a striking resemblance to the curves we show in our Figure 2 (20, Figs. 1c and 8). Koshland and his collaborators have studied the kinetics of such systems (10, 20). They concluded that the plateau indicates that the binding protein possess at least three ligand binding sites, and that the shape of curves of this type is due either to mixed negative-positive cooperativity in ligand binding, or to changes in the catalytic constants as a function of ligand (substrate) occupancy.

Further, Koshland (11) has also pointed out that if a system has been converted to the fully active form before the addition of substrate, then the subsequent binding of substrate will proceed without further conformational change and will follow a simple Michaelis-Menten pattern. On the basis of the contrasting curves we show in Figure 3, we suggest that under the condition of pH 5.5 light, the transport system is present in its fully active form, as indicated by the Michaelis-Menten kinetics. Protonation alone is not sufficient to convert it to this fully active form, since in the dark the intermediary plateau type of curve was obtained even when the medium was at pH 5.5. (The system was indeed protonated in the dark at pH 5.5, as is indicated by the analysis of rate of uptake versus proton concentration shown in Fig. 4.) We conclude that some other factor is operating in the light, which, together with protonation, brings about full activation. This other factor, might be the electric field imposed by a large membrane potential, which may only be achieved in the light (17, 18). It could also be some other factor differentiating an illuminated from a nonilluminated protoplast. In the absence of the light factor, ligand energy eventually brings about full activation of the system, as evidenced by the fact that, with increasing substrate concentration, the dark curve approaches that obtained in the light (Fig. 3, a and b).

CCCP reduces light uptake almost exactly to the dark level (Fig. 1a). This finding, we suggest, is not fortuitous. Under both conditions, uptake is proceeding unaided by the proton extrusion pump—in the former case because the latter is short-circuited by the proton conductor, and in the latter case because it may require light to activate the pump in our cells (cf. 15, 17, 18, 21). Yet dark uptake does not appear to be facilitated diffusion. The ratio of cytoplasmic to external concentration after 15 min of uptake (assuming cytoplasmic volume to be 5% of total protoplast volume) has frequently exceeded 5 when external concentration is below 1 mM. The net efflux of sugar shown in Figure 5 when protoplasts were transferred from pH 5.5 to pH 7 strongly suggests that accumulation above the external concentration had occurred at pH 5 in some cellular compartment.

Dark uptake was highly sensitive to pH, as is demonstrated by the immediate response of the unidirectional fluxes to changes in cis-pH (Fig. 5) as well as by the relationship shown in Figure 4. Further, dark uptake remained pH sensitive in the presence of CCCP (Fig. 1). The apparent conflict between this latter finding and that of van Bel (21) is probably resolved by the fact that initial flux was observed in our investigation whereas his measurements were made after some hours. This strict pH dependence even in the presence of CCCP, together with the very similar pH dependence of vacuolar uptake although the direction of the protonotive force is here reversed (14) are strong indications that the pH sensitivity may reflect a requirement of a constituent of the transport system for protonation and is not necessarily reflecting the energy input for transport. We have previously suggested, both in connection with dark uptake (8) and with uptake by vacuoles (7) that direct fueling by ATP may be one of the possible modes of energization of sugar transport in protoplasts as it is in bacteria (22). Whether dark uptake and light uptake are mediated by the
same system differently energized, as the pH sensitivity might suggest, or whether totally different systems are involved, must await further clarification.

LITERATURE CITED
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