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Changes in Envelope Permeability during Chloroplast Development *

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Summary. The permeability of the plastid envelope during the development of *Avena sativa* plastids was investigated by light scattering and uptake of various labelled compounds (malate, succinate, glutamate, α-ketoglutarate, citrate, glycine, sucrose). The results presented show that a primary event during greening is a change in permeability, thereby allowing an increased transport of metabolites across the membranes of very early etio-chloroplast stages. The results are discussed in view of an adaption of the plastid envelope permeability to the changing requirements of externally synthesized precursors and intermediates during development.

Introduction

Chloroplasts possess two membrane systems; the bounding double-membrane system of the plastid envelope, and the inner chlorophyll-containing lamellae. Although plastid lamellae have been investigated in detail much less is known of the structure and function of the plastid envelope.

The plastid envelope, separating the stroma from the cytosol, appears as two distinct membranes in electron micrographs. Freeze-etch studies have shown morphological differences between the two membranes (Bisalputra and Bailey, 1973). The outer membrane is thought to be freely permeable to metabolites of low molecular weight whereas the inner membrane functions as the metabolic barrier between the cytoplasm and the chloroplast (Heldt and Sauer, 1971).

In a recent study Cobb and Wellburn (1974) observed a primary light-dependent and cytoplasm-dependent increase in both total plastid protein and envelope associated protein during greening of *Avena sativa* laminae. The authors suggest from this that the envelope structure is altered during the process of greening.

This paper demonstrates that the alteration of the envelope structure during development is also expressed by an alteration of the permeability of the plastid envelope due to the changing requirements of externally synthesized precursors and intermediates of the plastid throughout its development.

Methods

Preparation of Etioplasts and Etio-chloroplasts

Nine-day old *Avena sativa* L. seedlings were grown in the dark or partially illuminated (metal halide lamp, Osram, 400 W, providing 6,000 lx) in the later stages of growth in a moist peat/permutite mixture at 25° C. Intact isolated etioplasts or etio-chloroplasts were prepared from the laminae by a loosely-packed Sephadex method (Wellburn and Wellburn, 1971), applying the following modifications: The laminae were homogenized in a medium containing 0.45 M sorbitol, 0.05 M MES, pH 6.1, 0.001 M $NaNO_3$, 0.001 M $MgCL_2$, and 0.001 M KH_2PO_4. The column medium contained 0.05 M HEPES, pH 7.3, instead of MES. The complete isolation was carried out within 45 min.

Measurement of Chloroplast "Transmission" Changes

Changes in "transmission" at 535 nm of plastid suspensions were measured at intervals from 1 s to 10 s. The transmission of a sample [2.5 ml of isolation medium with an osmolarity of about 0.5, containing plastids corresponding to a protein content of about 1 mg or to a plastid number from 0.8×10^6 (dark grown) to 0.3×10^6 (72 h light)] was recorded in a 1 cm cuvette. Small amounts of 2.0 M solutions of the substances tested were added to give a final concentration of 0.2 M and the change in transmission recorded in comparison to a control to which the same amount of aqua dest. was added.

Measurement of Penetration of Labelled Compounds across the Plastid Envelopes

a) Condition of Incubation. The incubations were carried out at 4° C in the same medium as that used for the Sephadex column,

* *Abbreviations:* HEPES = N-2-Hydroxyethylpiperazine-N'-2-ethane-sulfonic acid; MES = 2(N-Morpholino)ethane-sulfonic acid.

containing plastids corresponding to about 2 mg protein/ml. The final concentration of the substances tested was 5 mM.

b) Filtering Centrifugation. The penetration of labelled compounds across the plastid envelope was measured by filtering centrifugation (Klingenberg and Pfaff, 1967). The uptake of labelled compounds was determined by counting the sediment and 100 µl aliquots of the supernatant after rapid centrifugation of the plastids through an inert layer of silicone oil. The centrifugation was carried out with the Beckman minifuge 152 (15 s). Centrifuge tubes (0.5 ml) contained 50 µl $HClO_4$, 10%, 100 µl silicone oil (Type AR 150, Wacker Chemie, München, F.R.G.), and 200 µl suspension of plastids. Plastid spaces in the sediment were calculated from the amounts of tritiated water (water space) and of the non-permeating (^{14}C) sorbitol (sorbitol spece) to correct unspecific permeation into the inner membrane space. Dextran was used (2 mg/ml) to correct for medium adhering to the outer surface of the plastid (see Heldt and Sauer, 1971).

The amount of protein was measured according to Lowry *et al.* (1951).

The degree of integrity of the plastids was determined by phase contrast microscopy. About 70–90% of the plastids were found to be intact at the end of the different incubation times.

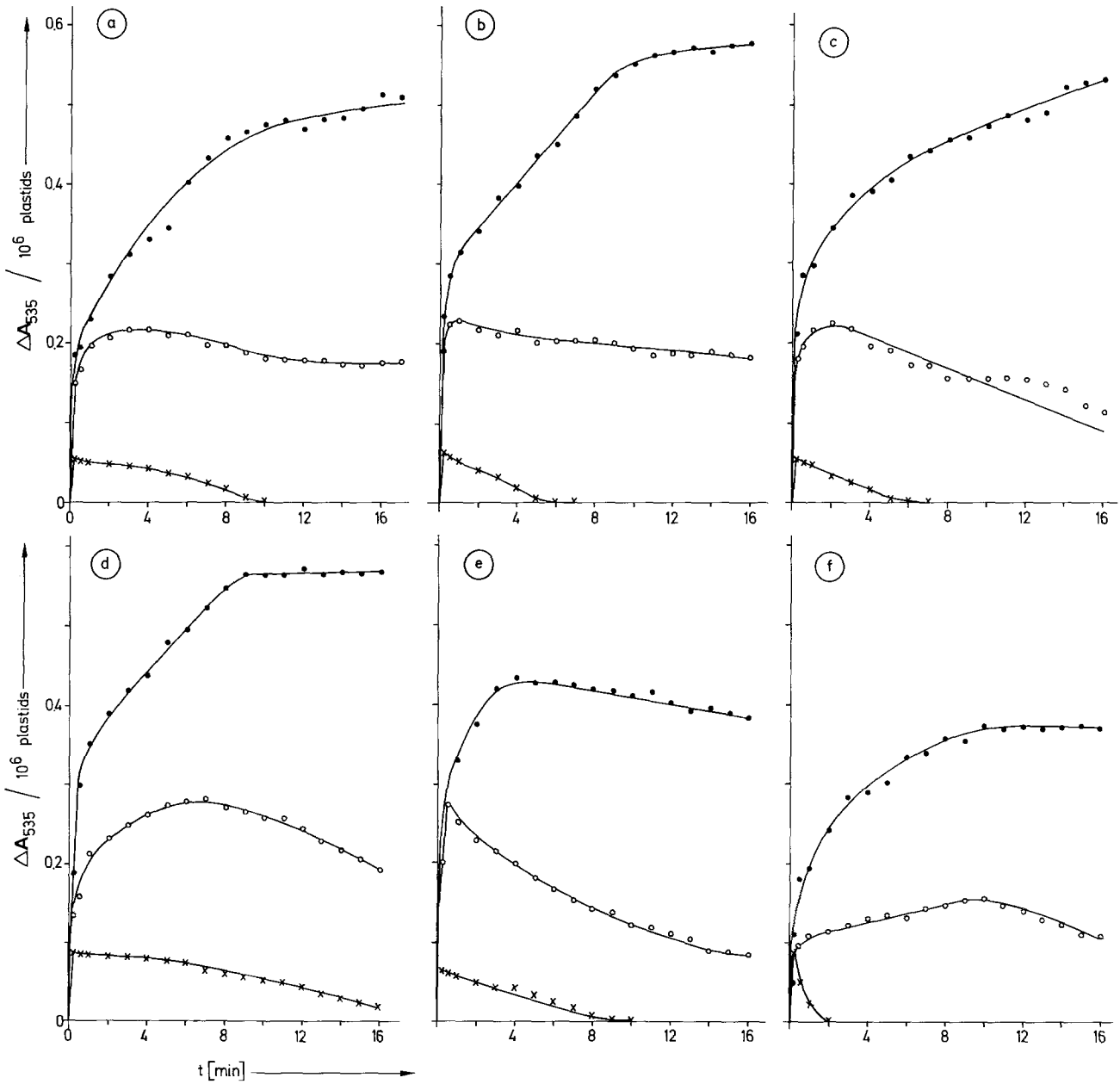

Fig. 1a–f. Kinetics of the changes in absorbance at 535 nm, called forth by the addition of glutamate (a), α-ketoglutarate (b), succinate (c), citrate (d), malate (e), and glycine (f) (final concentration 0.25 M)) to intact plastids. The osmolarity before addition was 0.5; ×—× etioplasts; ○—○ etio-chloroplasts (24 h light); ●—● etio-chloroplasts (72 h light)

Results

1. Osmotic Volume Changes and Light Scattering of Plastid Suspensions

With the light scattering technique changes in absorbance at 535 nm can be measured. Those are due to changes in particle volume (see Gimmler *et al.*, 1974). Intact plastids show an osmotic response upon addition of non-permeating compounds. This can result in shrinkage of the plastids (hypertonic solutions), or in swelling and in rupture of the plastid envelopes (hypotonic solutions). Permeating compounds only require a short time to have an effect. Consequently this method is applicable to measurement of the permeability of the plastid envelopes towards different compounds during the various stages of plastid development.

Fig. 1 a–f show the kinetics of change in apparent absorbance at 535 nm in the response to the different substances. Generally for etioplasts, there is a fast increase in absorbance upon addition of the different compounds which is followed by a slower decrease: The plastids then swell again to reach their primary

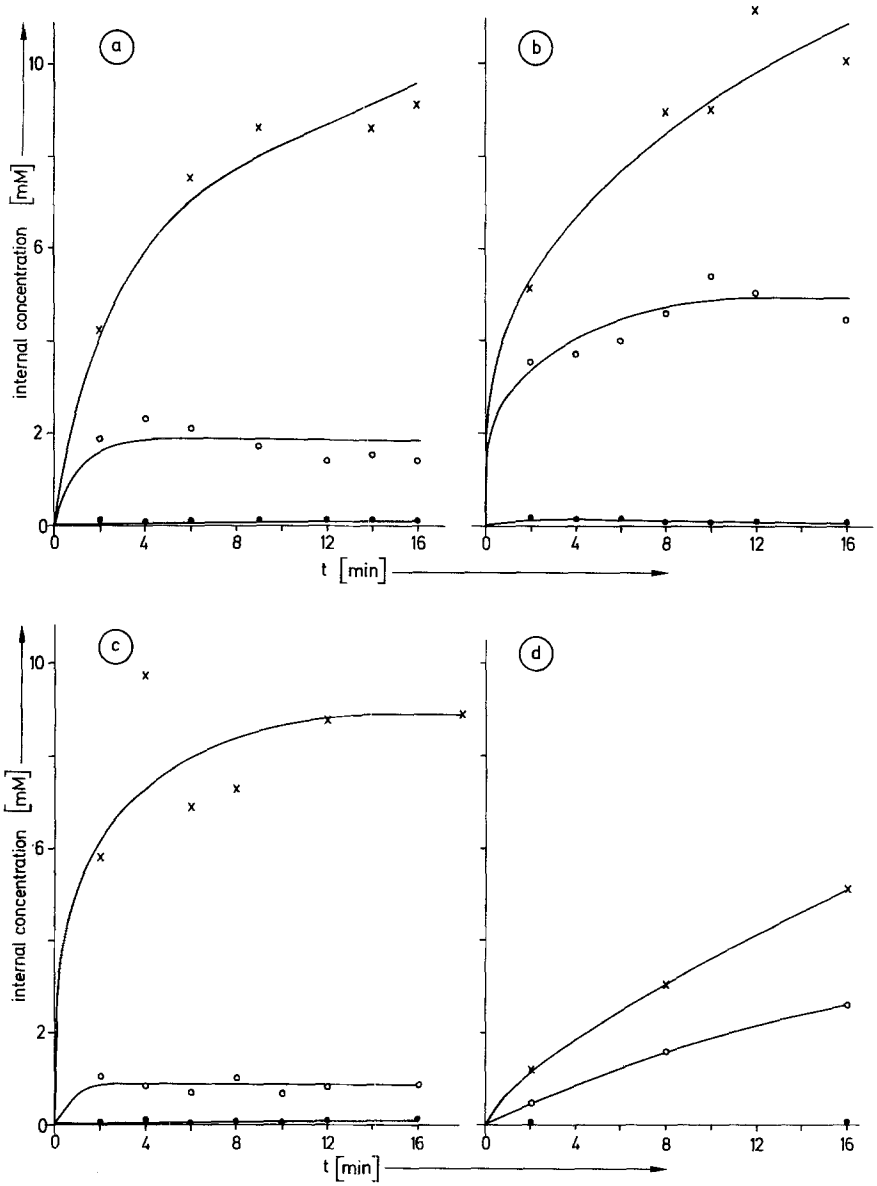

Fig. 2a–d. Uptake of radioactive labelled compounds [glutamate (a), glycine (b), succinate (c), sucrose (d)] into the sorbitol-impermeable space of plastids; ×—× etioplasts; ○—○ etio-chloroplasts (24 h light); ●—● etio-chloroplasts (72 h light); external concentration of the respective compounds 5 mM

volume (ΔA_{535} reaches zero). This means that these substrates permeate into the plastids.

Plastids that have developed for 24 h in the light show a more pronounced shrinkage upon addition of substrates; but invariably there is a decrease of absorbance after time. Later the plastids tend to swell again in order to reach the volume they had before treatment. In contrast to etioplasts the time required for this to take place is significantly longer.

The behaviour of plastids of 72 h light treated leaves is again different from those less well developed. An irreversible increase of absorbance occurs upon addition of all the substances tested with the exception of malate. After a certain time the absorbance tends to reach an elevated plateau.

It is also interesting to compare the effects educed by the substances tested. For etioplasts the time taken for swelling upon addition of glycine, succinate, and α-ketoglutarate was shorter than with glutamate, malate or citrate. While the effects of most substances are quite similar for the 24 and 72 h developmental stages malate is the exception. For this molecule swelling was observed in all developmental stages.

2. *Uptake of Labelled Compounds into Intact Plastids*

Figs. 2 and 3 show the uptake of various radioactive labelled compounds into the sorbitol-impermeable space of plastids. In Fig. 2a–d the internal concentration of glutamate, succinate, glycine, and sucrose is plotted as a function of time. Glutamate, glycine, succinate, and to a lesser extent, sucrose are found to be rapidly transported into the etioplasts, whereas after about 2 min etio-chloroplasts (24 h) show that there is a saturation at a low internal concentration with succinate (0.9 mM) and glutamate (2 mM) but glycine and sucrose accumulate better. Etio-chloroplasts of leaves that have been treated with light for 72 h show a very low uptake of glutamate, succinate, and glycine, but no uptake of sucrose at all.

Fig. 3 illustrates the average values for the uptake of malate, succinate, glutamate, α-ketoglutarate, citrate, glycine, and sucrose over the incubation time (16 min) into the sorbitol-impermeable space of different developmental stages of the plastids. The internal concentration of malate, succinate, glutamate, glycine, and sucrose in etioplasts exceeds the external concentration of the respective substrates (5 mM), whereas there is no accumulation of citrate and α-ketoglutarate. However, in etio-chloroplasts the ratio of internal to external concentration of the substances tested never exceeded unity. Malate, citrate, and glycine nearly equal the external concentration but there is a smaller uptake of sucrose and a poor uptake of glutamate, succinate, and α-ketoglutarate.

Into etio-chloroplasts (72 h) only certain amounts of malate, citrate, and α-ketoglutarate are incorporated. There is no uptake of glycine, succinate, glutamate, and sucrose.

Discussion

The results presented here show a change in the envelope permeability during the development of plastids. But there are quantitative differences between the substrates tested. Malate is readily taken up by all developmental stages. This is in good accordance with

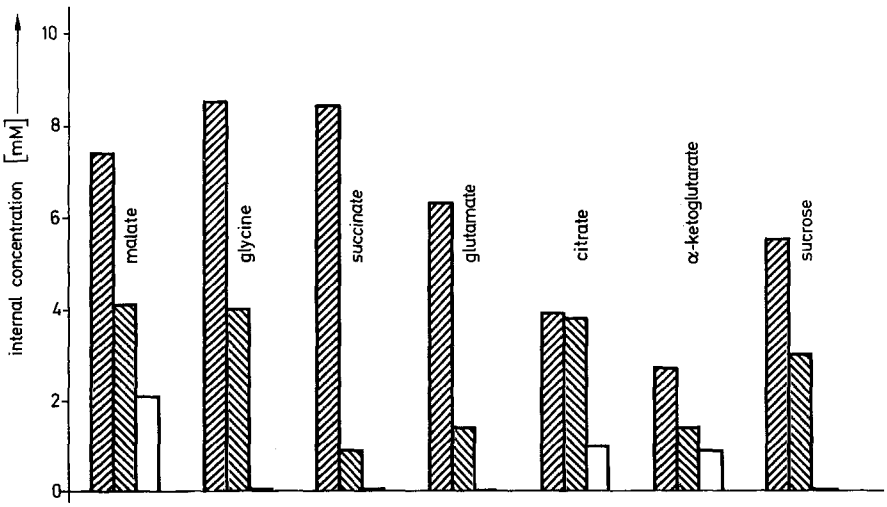

Fig. 3. Uptake of radioactive labelled compounds into the sorbitol-impermeable space of plastids; average values over a incubation time of 16 min; ▨ etioplasts; ▧ etio-chloroplasts (24 h light); □ etio-chloroplasts (72 h light); external concentration of the respective compounds 5 mM

the results of Heldt and Rapley (1970) and Gimmler *et al.* (1974) using isolated chloroplasts of spinach. The uptake of glutamate and succinate into the sucrose-impermeable space of spinach chloroplasts is relatively good compared to citrate. The reverse is true for Avena plastids that have been illuminated for 72 h, only an uptake of citrate is measurable. The results obtained with sucrose are also interesting. For spinach chloroplasts sucrose is known to be a non-permeating compound (see Gimmler *et al.*, 1974) but it permeates the envelope of early developmental stages of Avena plastids. Only envelopes of plastids that had a light treatment of 72 h were sucrose-impermeable. This means that in early developmental stages an exchange of disaccharides is possible between the intra- and extraplastidic spaces. In contrast the main transport is of DHAP and PGA in developed chloroplasts in addition to little transport of sugar phosphates (see Heber, 1974). This has the implication that sucrose should not be used as an osmoticum for the isolation of early developmental stages of plastids.

Also of interest is the relatively slow shrinkage of Avena etio-chloroplasts (72 h) as measured by light scattering. For spinach chloroplasts our own results and those obtained by Gimmler *et al.* (1974) show a significantly faster rate of change in absorbance. As the irreversible increase of absorbance indicates, plastid shrinkage is caused by the efflux of water from the plastid; consequently there must be a different resistance towards water efflux between spinach and Avena plastid envelopes. This must be a subject of further investigation to verify this.

In less developed plastids water flows back and swelling occurs when small molecules enter. This indicates that in early stages all compounds tested penetrate the plastid envelopes.

The results presented here are in accordance with the belief that plastids, although they contain genetic information, are not autonomous. Plastid differentiation requires the participation of both nuclear and plastidic DNA. The plastid envelope as a metabolic boundary between the extra- and intraplastidic space is important in cellular regulation. This regulation may be expressed by a change in the nature of the envelope. Cobb and Wellburn (1974) have demonstrated that the SDS-extractable envelope polypeptides in plastids isolated from greening tissue after a light treatment of 4 h increase 4 to 5 fold. As the envelope polypeptides are mainly of a higher molecular weight it is reasonable to suggest that those exclusive to the envelope are concerned with the permeability properties of the envelope system.

The results are furthermore in good accordance to those of Cockburn and Wellburn (1974) and Drumm and Margulies (1970), who showed that the synthesis of protein in isolated etio-chloroplasts seems to be associated with a change in the uptake of labelled leucine. The observed high incorporation level was maintained for up to 36 h. Thereafter, there was a decrease in the rate, correlating with a marked diminuition in the amount of chlorophyll accumulation in the illuminated leaves. This difference in the amount was shown only to be a function of the degree of development of the plastids and was not due to differences in amino-acid pool sizes.

From our results it can be concluded that the permeability of the plastid envelope may not only be a factor controlling the regulation of biosynthetic pathways once the plastid has reached maturity, but also adapts to the changing requirements of externally synthesized precursors and intermediates during development.

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