THE INFLUENCE OF CHILLING TEMPERATURE ALTERATION OF GLYOXYSOMAL SUCCINATE LEVELS ON ISOCITRATASE ACTIVITY FROM GERMINATING SEEDLINGS[1]

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Summary: Succinate inhibited isocitratase activity to a greater extent in isolated glyoxysomes of cold-sensitive cotton than in cold-tolerant castor bean seedlings. Exposure of either isolated glyoxysomes or intact seedlings prior to glyoxysome isolation to 5°C appeared to alter the permeability of the glyoxysomal membranes to succinate. Endogenous succinate accumulated in glyoxysomes from 5°C treated cotton seedlings but not in similarly treated castor bean seedlings.

Exposure of many plant species to near-freezing temperatures for transient periods during early germination and seedling establishment is inhibitory (2) and in some instances lethal (3). There have been a number of reports on metabolic (4,11,12) alterations which occur during or after such treatment.

In species utilizing lipids as a primary reserve for use during germination, one manifestation of low temperature (5°C) susceptibility (cotton) was apparent reduced levels of isocitratase (13). Significant reductions in isocitratase levels were not observed in cold-tolerant castor bean (13). However, whether such exposure affected enzymic activity or enzyme quantity in cold-susceptible species remained unclear (13).

The results presented in this report suggest that exposure of susceptible oil-storing seeds to chilling temperature during germination causes, in part, an

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alteration of glyoxysomal membrane permeability to succinate. As succinate accumulates in the glyoxysomes, it progressively inhibits isocitratase activity, thereby impeding the conversion of lipid to carbohydrate. The subsequent impairment of carbohydrate availability to the axis could thus contribute to reductions in seedling growth and viability.

MATERIALS AND METHODS

Fungicide-treated cotton (Gossypium hirsutum L. var. Coker 413) and castor bean (Ricinus communis L. var. Baker 296) seeds were germinated and exposed to chilling temperature (5°C) as previously described (13).

Glyoxysomal preparations were obtained by homogenizing 20-50g of tissue in 80-200 ml of a grinding medium initially described by Briedenbach and Beevers (1) and modified by Longo and Longo (9). Glyoxysomes were separated from mitochondria and proplastids by centrifugation in linear sucrose density gradients (1). For the exogenous succinate experiments, the glyoxysomal bands from a number of gradients were removed pooled, centrifuged at 11,000 xg for 15 minutes, and the resultant pellet resuspended in 5 to 10 ml of 35% sucrose.

For determination of the levels of certain endogenous organic acids by gas chromatography, glyoxysomes from 72 h germinated cotton and 96 h germinated castor bean (with either none or a 6 h exposure to 5°C prior to harvest) were isolated and purified as described above. Fractions containing 4 to 8 mg glyoxysomal protein were sonicated for two 30 second intervals before desiccating the samples by lyophilization. The resulting material was methylated similar to the

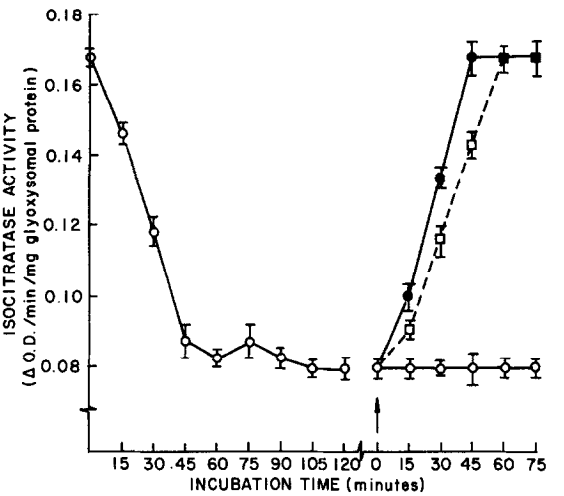

Figure 1. Isocitratase activity in isolated glyoxysomes from 4 day old cotton seedlings after the addition of 1mM succinate (o—o). After 2 hours, glyoxysomes were left in succinate (o—o) or incubated at 5°C for 0 (●—●) or 4 hours (□--□) before transfer (arrow) to succinate-free medium (see text).

procedure of Harvey et al. (8). Gas chromatographic analysis will be described in detail elsewhere (14).

Isocitratase was assayed according to Dixon and Kornberg (5). Protein was determined using the method of Lowry et al. (10). All experiments were repeated from 3 to 6 times. Standard deviations from the mean were calculated for all data.

RESULTS AND DISCUSSION

In preliminary experiments, a number of metabolites were individually added to the culture medium in order to determine their affects on cotton seedling growth and isocitratase levels (14). Of these, only succinate proved to be inhibitory, and when added at 5×10^{-3} M, duplicated the effects of chilling temperature (5°C) exposure (14).

Glyoxysomes were subsequently isolated from normally germinated (24°C, no succinate) cotton and castor bean seedlings,

Table 1. Isocitratase activities of cotton and castor bean glyoxysomes after a 45 min. incubation in the presence of added succinate. Data presented (average of 4 determinations) as the per cent inhibition compared to glyoxysomes incubated the same period in 35% sucrose only.

Succinate concentration (mM)	Percent inhibition of isocitratase activity	
	Cotton	Castor bean
1	13 ± 2.5	0
10	85 ± 1.4	0
25	97 ± 1.3	28 ± 3.4

suspended in 35% sucrose and challenged with varying concentrations of succinate for 45 minutes before determining isocitratase levels (Table 1). Isocitratase activity in glyoxysomes from the chill-susceptible species (cotton) was sensitive to increasing concentrations of succinate while the activity of isocitratase from the chill-resistant species (castor bean) was affected only at concentrations greater than 10 mM (Table 1).

Addition of succinate to isocitratase assays without preincubation (using sonicated glyoxysomes as enzyme source) indicated end product inhibition (14) similar to that reported for algal (6) and spinach (7) isocitratase.

The time course of inhibition of isocitratase in cotton glyoxysomes by 1mM succinate is shown in Figure 1. When succinate was removed from the incubation medium by centrifugation of the glyoxysomes from succinate-sucrose medium and resuspending in 35% sucrose, isocitratase activity was restored to initial levels within 45 minutes (Figure 1). If glyoxysomes were incubated at 5°C for 4 hours before placement in a

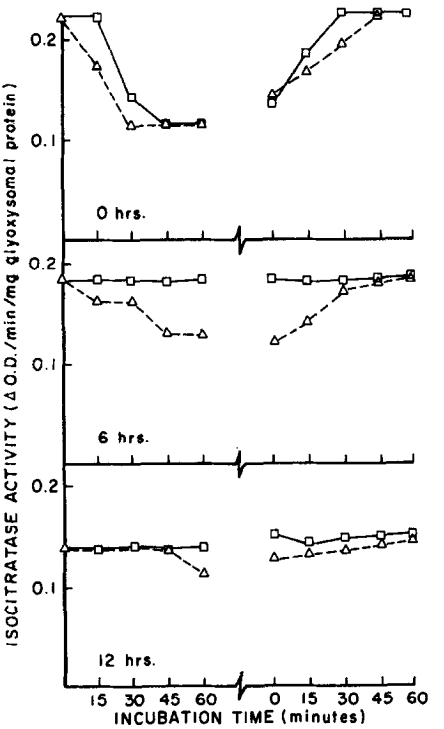

Figure 2. Isocitratase activity in isolated glyoxysomes of 4 day germinated cotton seedlings. Glyoxysomes incubated in 1mM (□--□) or 10 mM (Δ---Δ) succinate for 60 minutes (left) before placement (right) of glyoxysomes in succinate-free medium (see text). Seedlings incubated at 5C for 0, 6 or 12 hours before glyoxysomal isolation.

succinate-free medium, restoration of isocitratase activity was slightly retarded (Figure 1). Incubation of isolated enzyme for various periods at temperatures between 5° and 20°C indicated that the isocitratase from cotton was not cold labile (14).

The preceeding experiment suggested that once exogenous succinate was taken up by the glyoxysomes, exposure of the organelles to low temperature could enhance succinate retention (based on the slowed recovery of isocitratase activity - Figure 1). Thus, we conducted a reciprocal test in which intact seedlings were exposed to 0, 6 or 12 hours of

Table 2. Organic acid levels in glyoxysomes isolated after 0 or 6 hr exposure of seedlings to 5C. Cotton and castor bean seedlings were germinated at 24C for 72 and 96 hrs, respectively, before chilling. Data are means from 3 (castor bean) and 6 (cotton) determinations.

Source	Chilling period (hrs)	mg organic acid/mg glyoxysomal protein		
		citrate	malate	succinate
Castor bean	0	5.0 ± 0.5	1.1 ± 0.1	2.9 ± 0.3
Castor bean	6	9.9 ± 0.1	1.7 ± 0.3	1.9 ± 0.4
Cotton	0	5.2 ± 0.3	6.2 ± 0.8	5.7 ± 0.3
Cotton	6	12.0 ± 0.7	18.0 ± 1.9	110 ± 14

chilling (5°C) prior to the isolation of the glyoxysomes. The isolated glyoxysomes were incubated in isotonic sucrose containing 1 or 10 mM succinate (Figure 2). Isocitratase in glyoxysomes from normally (24°C) germinated seedlings proved susceptible to both succinate concentrations (Figure 2 - 0 hours), similar to the previous results (Table 1). However, with increasing low temperature exposure prior to glyoxysome isolation (6 or 12 hours - Figure 2), isocitratase activity was progressively less affected by exogenous succinate.

We concluded from these results (Figure 1 and 2) that exposure to chilling temperature was altering the permeability of the glyoxysomal membrane to succinate. Lyons et al. (12) have previously reported changes in plant mitochondrial membrane "flexibility" initiated by low temperature treatment. Further, Wade et al. (15) using cotton glyoxysomes and a spin labelling technique have suggested that chilling temperature affects sensitive species through membrane-associated processes.

Our in vitro results, which implied that succinate was inhibitory to isocitratase activity and that chilling temperature altered the permeability of glyoxysomal membranes to succinate, led us to hypothesize that exposure of susceptible species to chilling temperature prevents succinate from diffusing from the glyoxysome causing its accumulation, whereupon isocitratase activity would be inhibited. This hypothesis was tested directly by determining organic acid levels in glyoxysomes from seedlings of cotton or castor bean exposed to chilling temperature. As indicted in Table 2, endogenous succinate levels in cotton glyoxysomes increased 19 fold during a 6 h chilling period prior to isolation and analysis. Although malate and citrate increased 2-3 fold, these organic acids had no influence on isocitratase activity when added directly to assay mixtures (14). Other organic acids expected to be present in glyoxysomes were detected but were too low to be quantified at the attenuations used during gas chromatographic analysis (14). With castor bean, which represents a chill-resistant species, succinate failed to accumulate in the glyoxysomes after a 6 h exposure to 5°C (Table 2).

Succinate inhibits isocitratase activity in a noncompetitive fashion (6,7,14). Although the enzyme from cotton is more sensitive to lower concentrations of succinate than castor bean isocitratase (14), the principal difference in susceptibility to low temperature between cotton and castor bean appears to be the permeability of their respective glyoxysomal membranes to succinate.

By lowering isocitratase activity, lipid breakdown and subsequent carbohydrate formation is impeded (13). Coupled

with the inhibition of mitochondrial respiration (11) and the enhanced leaching of carbohydrates from the radicle (4), the primary source of energy and carbon skeletons is sufficiently decreased to hinder embryonic axis growth. In cold-tolerant species such permeability changes apparently do not occur under the conditions described here and normal metabolite flow is thus maintained.

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