

# The Effects of Octanoate and Oleate on Isocitrate Lyase Activity during the Germination of *Pinus pinea* Seeds<sup>1</sup>

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## ABSTRACT

The changes of isocitrate lyase levels with respect to the catabolism of triglycerides have been studied during the germination of *Pinus pinea* seeds. We studied the effects of octanoate, oleate, and inhibitors of protein synthesis on isocitrate lyase during germination. Pyruvate kinase, glucose-6-P-dehydrogenase, malate dehydrogenase, and isocitrate dehydrogenase were also assayed. Octanoate and oleate inhibited the isocitrate lyase activity, similarly to cycloheximide, chloramphenicol, and actinomycin, inhibitors of protein biosynthesis. This inhibitory effect is not specific but is strikingly evident with isocitrate lyase. This inhibition was not proportional to the concentration but was proportional to the chain length of oleate and octanoate.

Isocitrate lyase (E.C. 4.1.3.1.) is an enzyme of the glyoxylate cycle. In some organisms, this cycle provides a mechanism for the transformation of lipids into sugars (2, 4, 7, 11, 28). Although some Krebs cycle enzymes are components of the glyoxylate cycle, the key enzymes of the anaplerotic pathway are two: isocitrate lyase and malate synthase; the former begins the cycle and is responsible for its regulation (24). There are many data in the literature dealing with the regulation of IL,<sup>2</sup> but generally they concern either bacterial IL or *in vitro* experiments, carried out directly on the enzyme (8, 27). The *in vivo* activation of the enzyme by acetate in presence of proline, glutamate, or  $\alpha$ -aminobutyrate as a carbon and/or nitrogen source in certain bacterial species has been reported (20, 21).

As for the inhibition of the enzyme, the action of pyruvate and P-enolpyruvate or their derivatives is known (16, 17). In the angiosperms, the *de novo* synthesis of the enzyme appears during seed germination and generally is accompanied by high levels of lipids (6, 18, 22, 23). Thus "age" has been considered the controlling factor (10). Data about the action of some fatty acids on bacterial isocitrate lyase have been also reported. In some cases the fatty acids have an activating effect on IL; in other cases, oleate inhibits the synthesis of IL completely (19). In an attempt to provide biochemical data on lipid catabolism and IL activity in complex organisms such as the seeds of a

conifer, we have studied the changes of IL levels and compared them to the catabolism of lipids, particularly triglycerides. We have studied the *in vivo* effects of two fatty acids, octanoate and oleate, added to the culture medium during the germination of *Pinus pinea* seeds (29).

Enzymes of other metabolic pathways were also assayed as controls: PK and G6PDH, MDH and IDH. The effects of octanoate and oleate in the culture medium have been studied in comparison with the action of some inhibitors of protein biosynthesis. Moreover, a correlation between the chain length of the fatty acids studied and the IL activity has been established.

## MATERIALS AND METHODS

**Seed Germination.** Seeds of *Pinus pinea*, thoroughly rinsed in distilled water, were germinated on wet filter paper in Petri dishes at room temperature in diffuse daylight (experimental period March 1 to May 15). A proper amount of water was added to allow complete inhibition and normal germination. A group of seeds were germinated with octanoate and oleate at the studied concentrations; the pH of the medium was constant at about 7.0. Metabolic inhibitors were added to the culture medium on the 7th day of germination. At the desired period of germination the materials were collected and used for the determination of enzymes, proteins, and lipids.

**Lipid Extraction and Determination.** We have employed the method of Folch *et al.* (14) for the extraction of lipids. For the separation of the lipid classes we used TLC on silica gel as described by Malins and Mangold (25). When necessary, the fractions were permanently stained by spraying the plates with 50% (v/v) H<sub>2</sub>SO<sub>4</sub> containing 1.2% potassium bichromate. The triglyceride fraction was scraped from the plate, eluted with ethyl ether, and analyzed for fatty acid composition by gas-liquid chromatography of the methyl esters. The analysis was carried out at 170 C on a column 2.5 m in length 3.4 mm i.d. packed with 15% diethylene glycol succinate on 80 to 90 mesh Anakrom ABS. Areas under peaks were calculated by triangulation with respect to an internal standard.

**Preparation of Enzyme Extract.** The samples were homogenized with a medium 1.5 times their weight, containing 100 mM tris (pH 7.4), 2 mM EDTA, 10 mM mercaptoethanol, and Tween 80 (1%, v/v), in an Ultra-Turraz apparatus for 1 min at 0 C, according to Bartels *et al.* (1) and Firenzuoli *et al.* (12). The homogenate was subsequently centrifuged at 15,000 rpm at 0 C for 15 min, and the insoluble fraction was discarded. The supernatant fluid was recentrifuged at 45,000 rpm at 0 C for 45 min, and the clear supernatant was used for protein and enzyme determinations.

**Protein Determination.** The total protein of the enzyme extract was determined by the biuret method according to Beisenherz *et al.* (3). To avoid possible interference of pig-

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<sup>2</sup> Abbreviations: IL: isocitrate lyase; PK: pyruvate kinase; G6PDH: glucose-6-P-dehydrogenase; IDH: isocitrate dehydrogenase; MDH: malate dehydrogenase.

ments or lipid substances on the final color, the protein precipitate was washed with *n*-butyl alcohol before being dissolved in the cupric reagent.

**Enzyme Assays.** Isocitrate lyase activity was assayed at 25 C by the continuous optical method of Dixon and Kornberg (9) slightly modified by us (12) using a Beckman model DB spectrophotometer equipped with a thermoregulated cell holder and with a Sargent model SR recorder. The other enzyme activities were determined by optical tests based on the extinction change of pyridine nucleotides at 366 nm according to Bücher *et al.* (5).

**Material Used.** Tris, EDTA, enzymes, and coenzymes were

purchased from Boehringer and Soehne; Tween 80 and mercaptoethanol from Fluka AG, Buchs; substrates from Sigma Chemical Company or from Boehringer; all other products were obtained from Merck.

## RESULTS AND DISCUSSION

The levels of the enzymes are shown in Figure 1. The upper horizontal line indicates the controls, the second middle line shows the effect of octanoate, and the lower line the effect of oleate. Figure 1A<sup>1</sup> shows the change of the isocitrate lyase levels from the beginning of germination to the 20th day. The

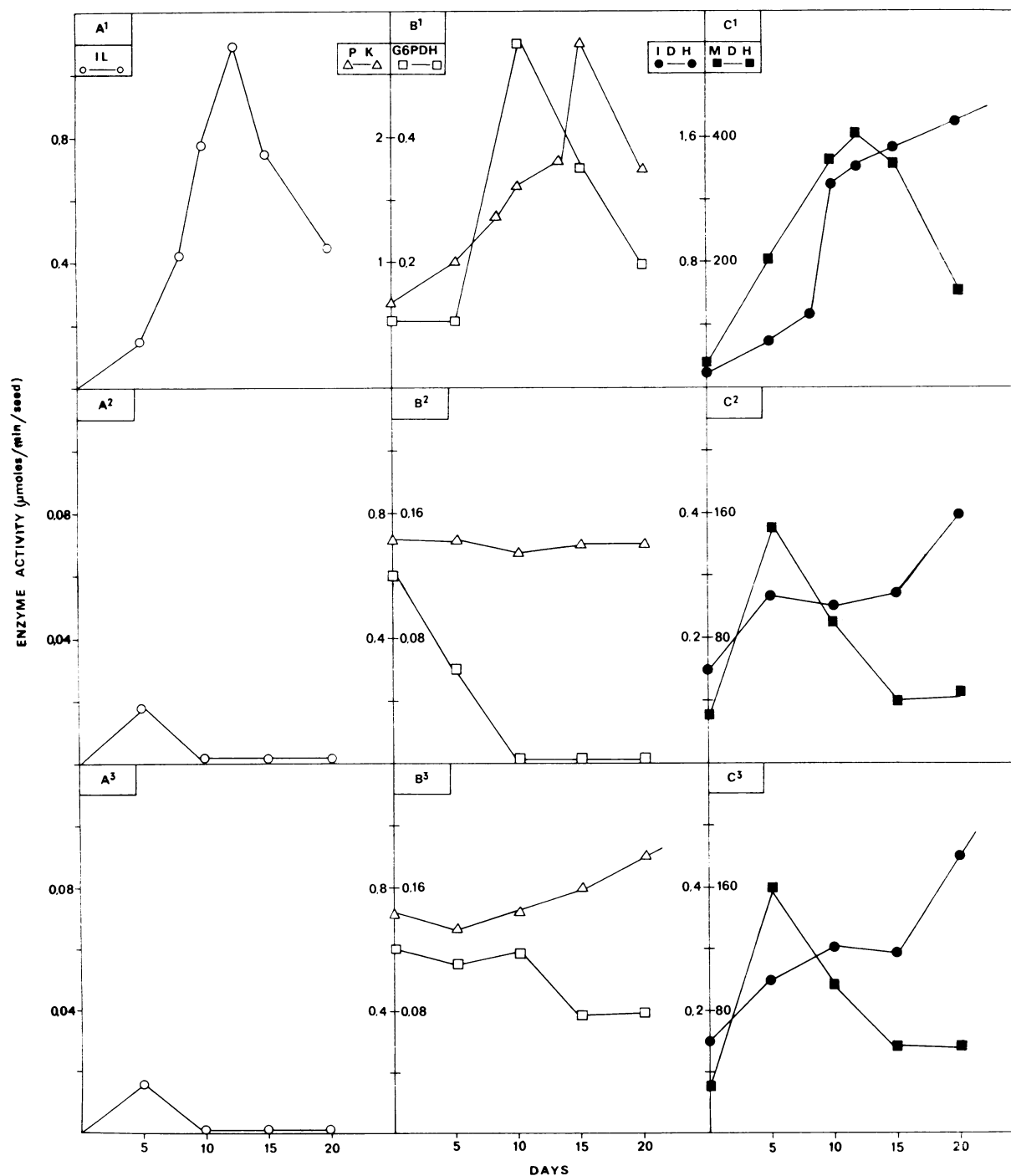


FIG. 1. Enzyme levels during germination of *Pinus pinea* seeds. In A<sup>1</sup>, B<sup>1</sup>, and C<sup>1</sup> are reported the levels of controls. In A<sup>2</sup>, B<sup>2</sup>, and C<sup>2</sup> there are the values of the enzyme when octanoate is added to the culture medium and in A<sup>3</sup>, B<sup>3</sup>, and C<sup>3</sup>, when oleate is added.

enzyme is absent at 0 time of germination, it has a maximum at the 12th to 14th day, and then it decreases. Figures 1A<sup>2</sup> and 1A<sup>3</sup> shows the effect of octanoate and oleate on isocitrate lyase; the values become appreciable only on the 5th day (0.018 units/seed); then the activity falls. Figure 1B<sup>1</sup> shows the G6PDH and PK activities in the controls. These enzymes are present at 0 time; the maximum of the activity for G6PDH is on the 10th day and for PK on the 15th day. Figure 1B<sup>2</sup> shows the octanoate effect on the two enzymes. It is evident that the levels of PK are constant during the germination, while 60 mM octanoate cancels the G6PDH activity in the first 10 days of germination. Figure 1B<sup>3</sup> indicates the oleate effect on the same enzymes. The action of the oleate is more moderate; in fact, for PK a small increase of activity is evident and the G6PDH inhibition is less marked. Figure 1C<sup>1</sup> shows the control levels for MDH and IDH. Both enzymes are present at 0 time. The IDH rises on the 20th day while MDH reaches its maximum at about the 12th day, then decreases. Figure 1C<sup>2</sup> shows the octanoate effect; the IDH outline is similar to the controls but the activity values are lower; the same holds for the MDH while its maximum is shifted to the 5th day. In Figure 1C<sup>3</sup> the oleate effect on the two enzymes is the same as that of the octanoate. Figure 2 shows the correlation between the concentration of octanoate and oleate added to the culture medium and the IL activity on the 5th day of germination. Octanoate 90 mM and oleate 10 mM inhibit the enzymatic activity completely. Inhibition is not proportional to concentration. Figure 3 indicates the correlation between the chain length of some fatty acids at constant concentration and the IL activity on the 5th day.

Table I shows the effects of octanoate (120 mM) and oleate (10 mM) when added to the culture medium on the 7th day. The table shows the IL activities on the 8th, 12th, 15th, and 20th days and the respective percentage of inhibition. Both fatty acids reach the same maximum percentage of inhibition at the 12th day. The table also shows the effects of various inhibitors of the protein biosynthesis on the same enzyme. These metabolic inhibitors were added to the culture medium on the 7th day; also in this case the percentages of inhibition increases in the course of time reach a maximum on 12th day of germination.

Figures 4 and 5 show the levels of oleic and linoleic acid (the fatty acids which are the major components of the triglyc-

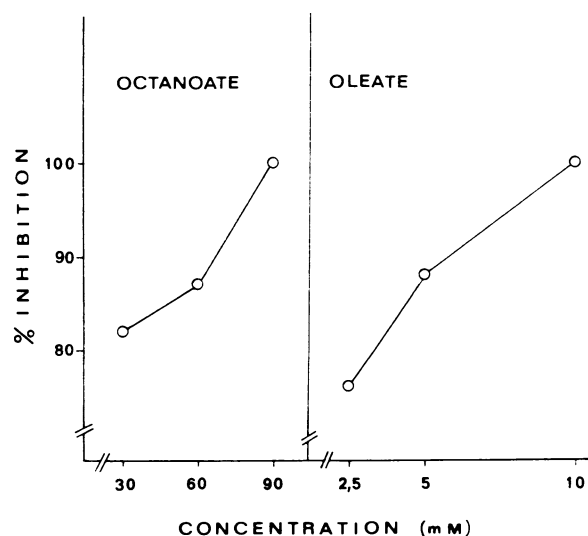


FIG. 2. Correlation between the concentration of octanoate and oleate and the levels of isocitrate lyase on the 5th day of germination.

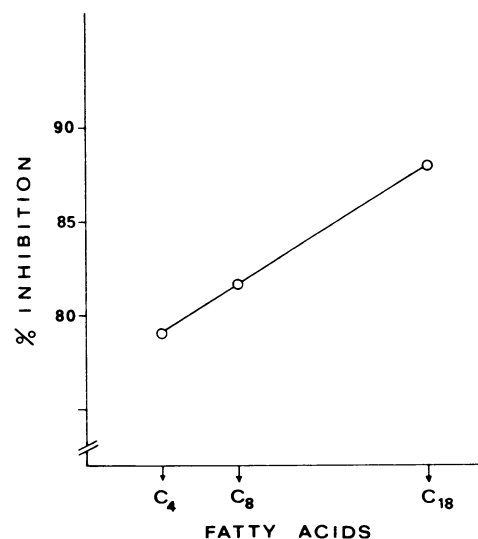


FIG. 3. Correlation between the chain length of fatty acids and isocitrate lyase (activity) levels on the 5th day of germination. (C<sub>4</sub>: butyrate; C<sub>8</sub>: octanoate; C<sub>18</sub>: oleate).

Table I. The Effects of Octanoate and Oleate and Some Metabolic Inhibitors on Isocitrate Lyase Activity

The fatty acids and metabolic inhibitors were added to the culture medium on the 7th day of germination.

Inhibitors	Isocitrate Lyase Activity after Germination for:			
	8 days	12 days	15 days	20 days
Control (no inhibitor)	0.47	1.1	0.75	0.45
Octanoate, 120 mM	0.12 (75) <sup>1</sup>	0.07 (94)	0.1 (87)	0.07 (85)
Oleate, 10 mM	0.10 (80)	0.07 (94)	0.17 (78)	0.09 (80)
Cycloheximide, 20 µg/ml	0.47	0.02 (98)	0.14 (81)	0.07 (84)
Chloramphenicol, 20 µg/ml	0.47	0.03 (97)	0.18 (76)	0.24 (47)
Actinomycin D, 20 µg/ml	0.24 (43)	0.02 (98)	0.05 (93)	0.04 (90)

<sup>1</sup> Numbers in parentheses are percentage of inhibition.

eride fraction), respectively, in controls and in seeds germinated either in 60 mM octanoate or in 5 mM oleate. The decrease of the studied fatty acids in the controls from zero time up to 15th day of germination is evident, while the octanoate and oleate added to the culture medium increase the levels of the two fatty acids studied, i.e., oleic and linoleic.

Octanoate and oleate effects on IL levels have been studied during *Pinus pinea* seeds germination. From our data it is evident that the increase in IL activity was inhibited after the 5th day of germination. This action is correlated to the concentrations of fatty acids studied. Moreover, it is evident that there is a direct proportion between the chain length of octanoate and oleate and their inhibition on the IL activity. The studies performed, comparatively, on G6PDH, PK, IDH, and MDH demonstrate a lower biosynthesis of these proteins. In fact, it is known that in the first days of germination there is generally a good increase of enzyme proteins (26). The maximum for the MDH is on the 5th day in the seeds germinated in presence of octanoate or oleate, similar to isocitrate lyase. This is in agreement with the formulation that MDH

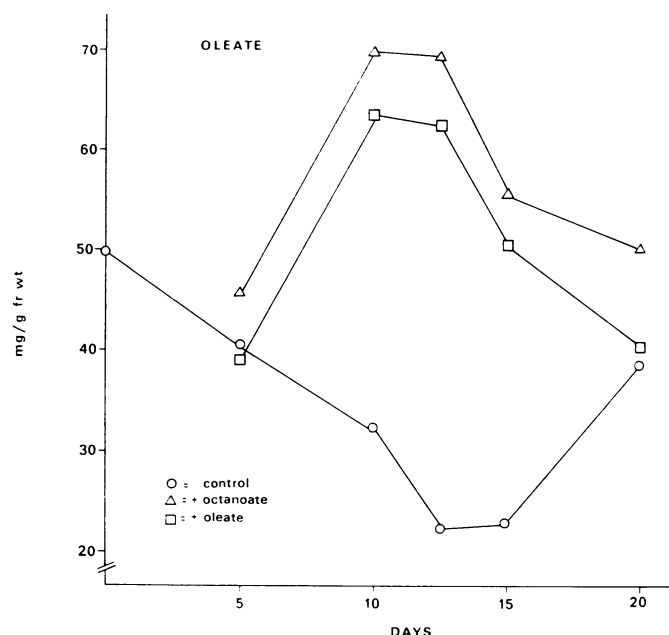


FIG. 4. Levels of oleate of the triglyceride fraction in the controls and in the seeds germinated in the presence of octanoate or oleate.

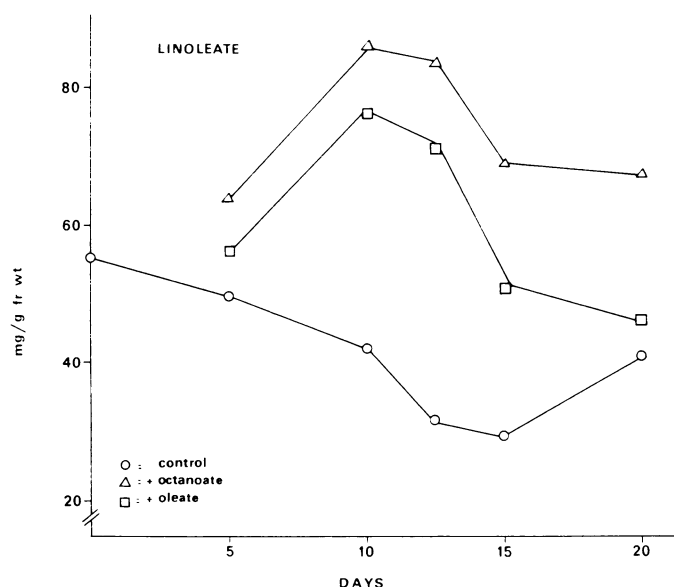


FIG. 5. Levels of linoleate of the triglyceride fraction in the controls and in the seeds germinated in the presence of octanoate or oleate.

participates in the Krebs cycle as well as the glyoxylate cycle. Particularly interesting is the sharp increase of IDH in the experiments performed in the presence of the two fatty acids; this observation might be taken as evidence that the seeds are still viable. With respect to controls, in the presence of octanoate there is no increase of PK, while a slight increase occurs in the presence of oleate. Octanoate and oleate not only stop the formation of G6PDH, but also inhibit the activity of this enzyme, indispensable to the development of the embryo (15). The inhibition of octanoate is complete and that of oleate is about the 50% with respect to zero time of germination.

It appears that octanoate and oleate particularly affect the

behavior of IL activity because the enzyme is absent at zero time and the two fatty acids inhibit its biosynthesis. This inhibition depends on the concentration and the chain length of the fatty acid (Fig. 3). Moreover, it is evident that the two fatty acids stop the *de novo* synthesis of the enzyme as strongly as the metabolic inhibitors. The maximum inhibiting effect is concomitant and can be found at about the 12th to 14th day of germination, when the *de novo* synthesis is greatest. A close correlation between the triglyceride content of the seeds and the formation and increase of IL activity is also confirmed. At the 1st day of germination we can notice that the octanoate and oleate effect is not remarkable either on IL activity or on the catabolism of the triglycerides. Subsequently, when the biosynthesis of the enzyme is inhibited, there is an increase of the triglyceride levels (oleic and linoleic acid). The TLC of total lipids in the controls shows the decrease of the triglyceride fraction during the germination, while in the presence of octanoate and oleate there is no effect on the lipid catabolism. All these results indicate that the free fatty acids added to the culture medium do not induce the IL biosynthesis during the germination of *Pinus pinea* seeds, but that octanoate and oleate inhibit the enzyme activity. Particularly, this inhibition depends on the concentration in the culture medium and on the chain length of the fatty acids.

It is interesting that the fatty acids tested directly on the enzyme in the reaction mixture are ineffective. We envisage that the block of the development of the embryo is due to the block and inhibition of G6PDH and IL activity, the former being an important enzyme for the synthesis of nucleic acids and NADPH (13, 30) and the latter being the key enzyme of the glyoxylate cycle, critical for the source of energy in the first days of germination. On the other hand, the experiments with the metabolic inhibitors demonstrated that the inhibition of the enzyme activity by the fatty acids studied (especially on IL and G6PDH) may be related to a complex mechanism which involves the inhibition of the protein biosynthesis.

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