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Comparative Studies on Chloroplast Development and Photosynthetic Activities in C_3- and C_4-plants.*

III. Studies on Activities of Some Enzymes Concerned with CO_2 Fixation Following the Chloroplast Development in Barley and Maize Leaves.

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This study is undertaken to investigate the activities of RuBP† carboxylase, Ru5P kinase, PEP carboxylase, NADP malate dehydrogenase, and aspartate aminotransferase following the chloroplast development in barley and maize leaves. RuBP carboxylase and Ru5P kinase are closely associated with the chloroplast development in barley and maize leaves, and both the activities are much higher in barley than in maize leaves. Activities of PEP carboxylase and NADP malate dehydrogenase are gradually increased throughout the chloroplast development in maize, but not in barley leaves. Activities of aspartate aminotransferase are not changed during the chloroplast development in both leaves of barley and maize plants. Activities of enzymes associated with the chloroplast development are gradually increased after 3-6 hours of lag phase, which is closely correlated with the chloroplast development and CO_2 fixation.

INTRODUCTION

Upon illumination to the etiolated leaf grown at a dark room chlorophyll accumulation (Virgin *et al.*, 1963; Boasson *et al.*, 1972), photosynthetic activities such as oxygen evolution (De Greef *et al.*, 1971; Oelge-Karow and Butler, 1971; Egnéus *et al.*, 1972) and CO_2 fixation (Tolbert and Gailey, 1955; Rhodes and Yemm, 1966; Tamas *et al.*, 1970), RuBP carboxylase, and Ru5P kinase (Benedict, 1973; Nato and Deleens, 1975; Hampp and Wellburn, 1976) occurred simultaneously with the ultrastructural changes of plastids. Upon illumination to the etiolated leaf prolamellar bodies formed at dark condition dispersed in the stroma. And by continuous illumination thylakoids were elongated and grana were formed in the chloroplast (Lee *et al.*, 1977). Consequently, chlorophylls were accumulated and CO_2 fixation rate was increased (Lee *et al.*, 1979). This

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† Abbreviation; RuBP: Ribulose-1, 5-bisphosphate, PEP: Phosphoenolpyruvate, Ru5P: Ribulose-5-phosphate.

study is performed to determine the relationships of the chloroplast development and CO_2 fixation with the activities of enzymes such as RuBP carboxylase and Ru5P kinase which are primary photosynthetic enzymes in C_3-plants, and PEP carboxylase, NADP malate dehydrogenase and aspartate aminotransferase which are primary photosynthetic enzymes in C_4-plants.

MATERIALS AND METHODS

Barley (*Hordeum vulgare* L., Fujinijyo) and Maize (*Zea mays* L., Golden Cross Bantham) were grown in a dark room at 25°C for one week. Leaf samples were prepared as in the previous paper (Lee *et al.*, 1977).

Preparation of crude enzyme

Two to three g of leaf tissues were thoroughly macerated in a chilled mortar and a pestle with a buffer solution containing 20 mM tricine, pH 8.3, 10 mM 2-mercaptoethanol, 0.2 mM EDTA, 16 g/*l* polyvinylpyrolidone, and a small amount of washed sands in a total volume of 10 m*l*. The resulting homogenate was then filtered through four layers of cheesecloth, centrifuged at 30,000 × g and 4°C for 20 min. The supernatant was used for enzyme assays.

Assay of RuBP carboxylase (EC. 4.1.1.39)

The reaction mixture contained 30 μmol tricine, pH 8.0, 10 μmol $MgCl_2$, 8 μmol 2-mercaptoethanol, 0.4 μmol RuBP, and 2.0 μmol $NaH^{14}CO_3$ in a volume of 200 μ*l*. Assays were initiated by addition of 50 μ*l* of the leaf extract and run 27°C for 2-3 min. The reaction was terminated by addition of 200 μ*l* of 25 % (V/V) acetic acid. Aliquotes were taken into a vial, and radioactivity was measured by a liquid scintillation counter (Kennedy, 1976).

Assay of PEP carboxylase (EC. 4.1.1.31)

The reaction mixture contained 30 μmol tricine, pH 8.0, 10 μmol $MgCl_2$, 8 μmol 2-mercaptoethanol, 1.0 μmol PEP, 1.0 μmol sodium glutamate and 2.0 μmol $NaH^{14}CO_3$ in a final volume of 175 μ*l*. Assays were carried out as given for RuBP carboxylase, except that 25 μ*l* of the extract was used (Kennedy, 1976).

Assay of NADP malate dehydrogenase (EC. 1.1.1.37)

The reaction mixture consisted of 25 mM tricine, pH 8.0, 1 mM EDTA, 0.5 mM oxaloacetate, 0.2 mM NADPH and 25 μ*l* of the extract in a total volume of 3 m*l*. The extract solution was incubated with 5 mM dithiothreitol and 5 mM $MgCl_2$ for 30-60 min in order to obtain the maximum activity of the enzyme. The reaction was initiated by addition of the leaf extract and activities were determined spectrophotometrically by oxidation of NADPH at 340 nm for 2-3 min (Hatch and Slack, 1969).

Assay of Ru5P kinase (EC. 2.7.1.19)

The reaction mixture consisted of 50 mM tricine, pH 7.6, 0.2 mM NADH, 1 mM ATP, 0.5 mM reduced glutathion, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM PEP, 5

units lactate dehydrogenase, 2 units pyruvate kinase, 2 units ribose-5-phosphate isomerase, and 0.2 m*l* of the extract in a volume of 1.5 m*l*. The reaction was started by the addition of 2 mM ribose-5-phosphate and activities were measured spectrophotometrically by oxidation of NADH at 340 nm for 2-3 min (Latzko *et al.*, 1970).

Assay of aspartate aminotransferase (EC. 2. 6. 1. 1)

The raction mixture contained 20 μmol tricine, pH 8.0, 100 μmol neutralized L-aspartate, 200 μg NADH, 20 μmol α-ketoglutarate, 4 units malate dehydrogenase and 25 μl of the extract in a final volume of 3 m*l*. Activities were determined spectrophotometrically by oxidation of NADH at 340 nm for 2-3 min (Sizer and Jenkins, 1962).

All enzyme assays were linear with respect to enzyme concentration and time, and showed no net activity in controls run without substrates. Protein was determined by using the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Activities of five enzymes concerned in primary CO_2 fixation following the chloroplast development in barley and maize leaves are given in Tables 1 and 2.

Table 1. Enzyme activities following the chloroplast development in barley leaves.

	Illumination					Green-house
	0 hr	3 hr	6 hr	18 hr	24 hr	
Protein (mg/g f.w.)	6.21	7.63	5.19	4.98	7.67	8.76
Enzyme	(μmol/mg protein/hr)					
RuBP carboxylase	7.25	6.91	8.25	11.22	16.91	38.42
PEP carboxylase	4.34	4.24	5.56	3.78	3.79	1.81
NADP malate dehydrogenase	6.73	7.54	7.25	—	7.46	6.56
Aspartate aminotransferase	19.80	18.69	22.06	17.02	18.23	10.43
Ru5P kinase	6.74	7.18	9.85	11.01	10.25	10.34

Table 2. Enzyme activities following the chloroplast development in maize leaves.

	Illumination					Green-house
	0 hr	3 hr	6 hr	18 hr	24 hr	
Protein (mg/g f.w.)	18.14	16.98	16.25	16.11	14.30	17.70
Enzyme	(μmol/mg protein/hr)					
RuBP carboxylase	1.69	1.64	1.76	2.22	2.64	3.31
PEP carboxylase	9.99	10.67	10.30	12.69	14.23	10.41
NADP malate dehydrogenase	6.49	7.25	7.86	9.71	10.03	7.06
Aspartate aminotransferase	22.69	24.53	18.58	21.83	23.20	20.88
Ru5P kinase	3.66	4.31	4.40	5.04	5.41	5.58

RuBP carboxylase and Ru5P kinase activities are increased in barley and maize leaves following the chloroplast development and are much higher in barley than in maize leaves throughout the chloroplast development, as shown by other workers (Benedict, 1973; Nato and Deleens, 1975; Hampp and Wellburn, 1976). RuBP carboxylase and Ru5P kinase were located in the chloroplasts of vascular sheath cells in C_4-plants and mesophyll cells in C_3-plants (Latzko and Gibbs, 1968; Slack *et al.*, 1969; Hatch and Kagawa, 1973). Moreover, RuBP carboxylase composed of two kinds of subunits with different molecular weight and amino acid composition (Rutner and Lane, 1967). The large subunit is coded by chloroplast DNA (Chan and Wildman, 1972) and synthesized by chloroplast ribosomes (Blair *et al.*, 1973). In these results the enzyme activities are gradually increased as the chloroplast development after the stage at which thylakoids are elongated and grana are initiated to form by the illumination to the etiolated leaves. Therefore, we may consider that RuBP carboxylase and Ru5P kinase are closely associated with the chloroplast development. On the other hand, PEP carboxylase is present in C_3-plants as well as in C_4-plants, but the physical property and biochemical function of this enzyme in C_4-plants are very different from those of C_3-plants. In C_4-plants the enzyme catalyses the reaction of carbon dioxide fixation under light condition, but can not in C_3-plants (Cooper and Wood, 1971; Ting and Osmond, 1973 a, b; Coombs, 1976). In the present results, the enzyme activities are also very different between barley and maize leaves. The enzyme activities are much higher in maize than in barley leaves and are increased throughout the chloroplast development in maize leaves, but not in barley. Therefore, it can be suggested that PEP carboxylase is associated with chloroplasts in C_4-plants, but not in C_3-plants.

Carbon dioxide accepted by PEP forms oxaloacetate and then this is transformed into malate by presence of NADP malate dehydrogenase whose activities are high in maize plants, malate former (Hatch and Slack, 1969; Gutierrez *et al.*, 1974). Moreover, the enzyme associates with chloroplasts of mesophyll cells in maize leaves (Slack *et al.*, 1969). However, aspartate aminotransferase is located in cytoplasm in mesophyll cells, whereas in vascular sheath layers the enzyme is located in mitochondria in aspartate former plants (Hatch and Mau, 1973). The present results show that NADP malate dehydrogenase activities are increased in maize leaves, but aspartate aminotransferase activities are not changed in maize as well as barley leaves throughout the chloroplast development. In the previous paper (Lee *et al.*, 1979), $^{14}CO_2$ was much incorporated into aspartate rather than into malate at an early stage of the chloroplast development in maize leaves, whereas with the further chloroplast development $^{14}CO_2$ was gradually fixed into malate more than into aspartate as well as mature leaves. Hence, it may be concluded that CO_2 tends to be incorporated into aspartate rather than into malate at an early stage of the chloroplast development because NADP malate dehydrogenase is closely associated with the chloroplast development but aspartate aminotransferase is not.

Generally, enzyme activities associated with the chloroplast development

exhibit 3-6 hours of lag phase and thereafter gradually increased with the further chloroplast development, which is closely correlated with the chloroplast development and carbon dioxide fixation.

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