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# Comparative Studies on Chloroplast Development and Photosynthetic Activities in $C_3$- and G-plants

## II. Studies on Carbon Dioxide Fixation in Barley and Maize Leaves

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This study was undertaken to investigate the fixation patterns of carbon dioxide and chlorophyll accumulation following the chloroplast development by illumination to the etiolated leaves of barley and maize seedlings grown in the dark for a week. CO, fixation rate was remarkably increased after 3 hours illumination in barley and maize leaves. These results were very closely correlated with differentiation of grana and chlorophyll accumulation. At an early stage of the chloroplast development in barley leaves, $CO_2$ fixation patterns were not different from those in leaves cultured in a green house. In maize leaves, however, $^{14}C$ was much incorporated into aspartate at an early stage of the chloroplast development in the leaves illuminated for 3 hours to the etiolated plant, and $^{14}C$ was much incorporated into malate at later stages of the chloroplast development as in the leaves cultured in a green house.

## INTRODUCTION

It is well known that the evolution of the photosynthetic apparatus from its simplest form in the green photosynthetic bacteria to the elaborate system of green plants is accompanied by the evolution of a more complicated pigment structure. Moreover, chloroplast is differentiated from its simplest system to very complicated system even in the same higher green plants. But the chloroplasts of vascular bundle sheath cells are devoid of grana even in mature leaves of maize seedlings. In the previous report (Lee et al., 1977), prolamellar bodies were formed in the etioplast of barley and maize leaves grown at a dark room for a week. These bodies were completely dispersed into lamellae in the chloroplasts of barley leaves illuminated by 1,000 ft-c of light for 3 hours after 6 days of dark germination, while in the chloroplasts within mesophyll cells of maize leaves the bodies still persisted during 18 hours under the same light condition and could not be observed in the chlo-

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roplasts of parenchyma vascular bundle sheath cells illuminated for 6 hours. Grana were formed at the chloroplasts of chlorenchyma cells in barley leaves and of mesophyll cells in maize leaves, but not at the chloroplasts of vascular bundle sheath cells in maize leaves. Ultrastructural aspects of chloroplasts within parenchyma vascular bundle sheath cells of maize leaves were similar to those of chloroplasts within chlorenchyma cells of barley at an early stage of the chloroplast development.

On the other hand, phosphorylated compounds are synthesized as initial photosynthetic products (Quayle *et al., 1954;* Calvin and Bassham, 1962) and compounds such as glycine, serine, phosphoglycolate and glycolate are much synthesized as photorespiratory products in $C_3$-plants (Tolbert, 1971; Kisaki *et al.,* 1972; Zelitch, 1973). While in $C_4$-plants dicarboxylic acids are synthesized as initial photosynthetic products (Kortschak et al., 1965; Hatch and Slack, 1966 ; Slack and Hatch, 1967) and compounds related to the glycolate pathway are not much synthesized because of low photorespiration (Jackson and Volk, 1970 ; Osmond and Harris, 1971). Moreover, $C_4$-plants can be divided into two groups, malate formers such as Zea *mays* and *Sorghum sudanense* in which malate is preferentially labelled on exposure to $^{14}CO_2$, and aspartate formers such as *Amaranths edulis, Atriplex spongiosa* and *Panicum miliaceum* in which aspartate is preferentially labelled on brief exposure to $^{14}CO_2$ (Downton, 1970; Edwards and Gutierrez, 1972).

The aim of the present studies was to investigate the patterns of $CO_2$ fixation in barley and maize leaves following the chloroplast development.

## MATERIALS AND METHODS

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

After exposure to light (ca. 1,000 ft-c), leaves were detached by cutting under water to expose to $^{14}CO_2$ atmosphere in the light or in the dark for 3 minutes. The detached first foliage leaves were placed vertically in a fixation chamber and the bases of leaves were immersed into water to prevent from shortage of water during $^{14}CO_2$ fixation. The light intensity at the leaf surface was about 2,500 ft-c. $^{14}CO_2$ was generated in the CO, fixation chamber by pouring 50 % (v/v) lactic acid into $NaH^{14}CO_3$ solution (65 $\mu$Ci/ml) and CO, concentration was adjusted to about 0.03 %.

After feeding of $^{14}CO_2$ for 3 minutes, the leaves were transferred to boiling ethanol (70 %, v/v). Then they were extracted in sequence with boiling ethanol (50 %, v/v) and boiling water. The extracts were concentrated under reduced pressure in a rotary evaporator below 40°C and passed through a column of Dowex-50 (H+) resin. And the fraction retained on the column was eluted with 2N $NH_4OH$. This fraction was used for amino acid analysis by paper chromatography (phenol : water=4 : 1, v/v). The effluent from the Dowex-50 (H+) column was passed through a column of Dowex-1 ($CH_3COO^-$) resin for organic acid analysis by Zelitch's method (Zelitch, 1965). The frac-

tion which was not adsorbed on the column was sugar. Radioactivity was measured by a liquid scintillation counter (Beckman Instrument Ltd., LS-250). Chlorophyll was measured by Comar's method (Comar and Zscheile, 1942).

Barley and maize were grown by an ordinary method in a green house as control to compare the $CO_2$ fixation pattern.

## RESULTS

### $CO_2$ assimilation

The rate of $CO_2$ assimilation was not increased both in light and dark fixation until 1 hour illumination stage of etiolated first foliage leaves of barley and maize plants. The rate of light and dark fixation in maize leaves was remarkably increased after 3 hours illumination stage, while the rate of dark fixation in barley was not increased throughout the chloroplast development. $CO_2$ assimilation rate was about 2 times higher at light fixation and 2-9 times higher at dark fixation in maize leaves than in barley leaves. However, the rate of light fixation was a little higher in barley leaves than in maize leaves cultured in a green house (Table 1). The rate of CO, assimilation and enzyme activities concerned in photosynthesis are decreased at aged leaves (Khanna and Sinha, 1973; Kennedy, 1976). It is therefore considered that the rate of CO, assimilation in the control maize leaves was due to aging of the first foliage leaves at this stage in green house cultivation.

**Table 1.** Total activity of $^{14}C$ fixed during 3 minutes exposure to $^{14}CO_2$ following the chloroplast development (cpm/mg. fr. wt.).

| Illumination | Fixation | Barley | Maize | Maize /Barley |
|---|---|---|---|---|
| 0 hour | Dark | 4 | | 0.3 |
| | Light | 10 | 2 | 0.2 |
| 1 hour | Dark | 6 | 14 | 2.3 |
| | Light | 15 | 30 | 2.0 |
| 3 hours | Dark | 29 | 129 | 4.5 |
| | Light | 400 | 650 | 1.6 |
| 6 hours | Dark | 23 | 106 | 4.6 |
| | Light | 600 | 1100 | 1.8 |
| 9 hours | Dark | 29 | 125 | 4.3 |
| | Light | 1500 | 2300 | 1.5 |
| 18 hours | Dark | 23 | 164 | 7.1 |
| | Light | 2500 | 4800 | 1.9 |
| 24 hours | Dark | 38 | 278 | 7.3 |
| | Light | 3900 | 6100 | 1.6 |
| Green house | Dark | 51 | 449 | 8.8 |
| | Light | 5700 | 4500 | 0.8 |

### Distribution of $^{14}C$ incorporated into sugar, organic acid and amino acid fractions

#### I) *Barley*

As in the previous paper (Lee et *al.*, 1977), grana formation initiated at the chloroplasts of the leaves illuminated for 3 hours. In this stage $^{14}C$ was incorporated 21.7 % into a sugar, 44% into an organic acid and 33.2 % into

an amino acid fraction, respectively. As successive illumination to the etiolated leaves, the incorporation rate of $^{14}C$ was increased in a sugar fraction following the chloroplast development, while decreased gradually in organic acid and amino acid fractions. By the illumination for 24 hours, $^{14}C$ was incorporated 46.3 % into a sugar fraction, 23.2 % into an organic acid fraction and 29.2 % into an amino acid fraction, respectively. This result was the same as in the leaves cultured during the same period in a green house. $^{14}C$ was incorporated 42.3 % into a sugar, 24.4 % into an organic acid and 32.7 % into an amino acid fraction, respectively in the leaves cultured in a green house (Fig. 1).

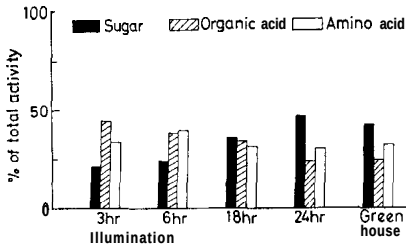


**Fig. 1.** Proportion of total $^{14}C$ incorporated into sugar, organic acid and amino acid compounds in alcohol extract of barley leaves during 3 minutes photosynthesis following the chloroplast development.

2) *Maize*

The proportion of total $^{14}C$ fixed was 4.5 % in a sugar, 35.5% in an organic acid and 59.4 % in an amino acid fraction, respectively in the leaves of 3 hours illumination stage. In this stage of the chloroplast development total $^{14}C$ fixed was incorporated much more into an amino acid fraction than any other two fractions. But following the chloroplast development, $^{14}C$ incorporated into a surgar fraction was gradually increased. Generally, $^{14}C$ was much more incorporated into organic acids than amino acids with the chloroplast development. In the leaves of 24 hours illumination stage, the rate of total $^{14}C$ fixed was 41.7 % in a sugar, 28.8 % in an organic acid and 27 % in an amino acid fraction, respectively. This was the same tendency as in the

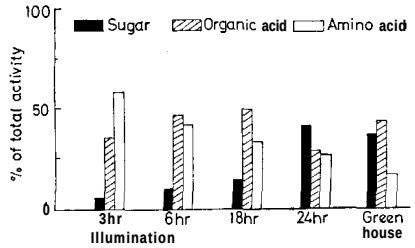


**Fig. 2.** Proportion of total $^{14}C$ incorporated into sugar, organic acid and amino acid compounds in alcohol extract of maize leaves during 3 minutes photosynthesis following the chloroplast development.

leaves cultured in a green house (Fig. 2).

**Proportion of $^{14}C$ incorporated into photosynthetic intermediates of each fraction**

$^{14}C$ was distributed as a proportion of 6. 6 % in serine+glycine, 4. 8 % in phosphoglycolate and 9 % in glycolate in barley leaves of 3 hours illumination stage, but the proportion of total $^{14}C$ incorporated into aspartate and malate which are primary photosynthetic intermediates in $C_4$-plants, was 7.1% and 8.5 %, respectively. In maize leaves illuminated for the same period as barley leaves, $^{14}C$ incorporated into aspartate and malate was 41.3 % and 22.1%. However, $^{14}C$ was incorporated 2.8 % into serine+glycine, 2.2 % into phosphoglycolate and 3.4% into glycolate. In barley leaves illuminated for 6 hours, $^{14}C$ was incorporated 17.3 % into serine+glycine, 4.6 % into phosphoglycolate, 10.4 % into glycolate, which were 32.3% of total fixed $^{14}C$, but 5 % into aspartate and 12.5 % into malate. The same tendency was observed in leaves illuminated for 18 hours and 24 hours, and in the leaves cultured in a green house (Table 2).

Table 2. Proportion of total $^{14}C$ incorporated into individual compounds following the chloroplast development in barley leaves (% of total $^{14}C$ fixed).

| Metabolic intermediate | Illumination 3 hrs | 6 hrs | 18 hrs | 24 hrs | Green house |
|---|---|---|---|---|---|
| Aspartate | 7.13 | 5.00 | 3.39 | 4.69 | 2.68 |
| Glutamate | 1.72 | 1.11 | 0. 70 | 0.90 | 0.67 |
| Serine+Glycine | 6.60 | 17.27 | 12.99 | 11.05 | *16.00* |
| Alanine | 13.50 | | 12.10 | 11.39 | *lo.* 58 |
| Other amino acids | 4.26 | 11.10 2.09 | 1.62 | 1.19 | 2.74 |
| | 33.21 | 36.57 | 30.80 | 29.23 | 32.67 |
| Glycolate | 9.03 | 10.36 | 9.61 | 4.66 | 9.59 |
| Malate | 8.48 | 9.60 | 5. 28 | 4.74 | 1.53 |
| Citrate | 19.27 | 12.49 | 11.10 | 8. 28 | 8.30 |
| Phosphoglycerate | *1.81* | 0.87 | 1. 50 | 1.12 | 0. 59 |
| Phosphoglycolate | *4.82* | 4. 63 | 6. 12 | 4. 17 | 4.27 |
| Other organic acids | *0. 60* | 0.14 | 0.15 | 0. 24 | 0.11 |
| | 44.01 | 38.09 | 33.76 | 23.21 | 24.39 |
| Sugar | 21.70 | 24.40 | 34.57 | 46.27 | 42.26 |
| Residue | 1.08 | 0.94 | 0.87 | 1. 29 | 0.68 |

In maize leaves illuminated for 6 hours, its proportion was 24.8 % in aspartate and 29.7% in malate. Generally, there was no characteristically different pattern of $^{14}C$ incorporation in each stage of the chloroplast development compared with that in leaves cultured in a green house (Table 3). However, at an early stage of the chloroplast development $^{14}C$ was much more incorporated into aspartate than into malate in maize leaves (Table 3).

**Chlorophyll accumulation**

Chlorophyll was accumulated linearly after 3 to 6 hours lag phase in the etiolated leaves of barley and maize seedlings as the chloroplast development.

**Table 3.** Proportion of total $^{14}C$ incorporated into individual compounds following the chloroplast development in maize leaves(% of total $^{14}C$ fixed).

| Metabolic intermediate | Illumination 3 hrs | 6 hrs | 18 hrs | 24 hrs | Green house |
|---|---|---|---|---|---|
| Aspartate | 41.34 | 24.81 | 12.37 | 12.10 | 4. 56 |
| Glutamate | 4.37 | 3.60 | 3.03 | 1.25 | 0. 78 |
| Serine +Glycine | 2.81 | 4.62 | 5. 98 | 3. 72 | 2. 98 |
| Alanine | 8.96 | 7.90 | 11.98 | 8. 77 | 8. 24 |
| Other amino acids | 1.88 | 1. 54 | 1.37 | 1.14 | 0.76 |
| | 59.36 | 41.93 | 34.73 | 26.98 | 17.32 |
| Glycolate | 3.40 | 3.59 | 5. 86 | 3. 34 | 5. 45 |
| Malate | 22.07 | 29.74 | 33.58 | 13.52 | 21.39 |
| Citrate | 5.99 | 8.28 | 7.72 | 4.05 | 13.37 |
| Phosphoglycerate | 1.55 | 2. 27 | 1. 54 | 4. 26 | 1.44 |
| Phosphoglycolate | 2. 16 | 2.58 | 1. 16 | 3.05 | 2.01 |
| Other organic acids | 0.36 | 0.31 | 0.14 | 0.54 | 0.11 |
| | 35.53 | 46.77 | 50.00 | 28.76 | 43. 77 |
| Sugar | 4. 52 | 9. 60 | 14.13 | 41.66 | 37.69 |
| Residue | 0.59 | 1. 70 | 1.14 | 2.60 | 1.22 |

The amount of chlorophyll was higher in barley than in maize leaves (Fig. 3). Grana were not well developed and chlorophyll b was deficient in the chloroplasts of vascular bundle sheath cells in maize leaves. But grana were present and chlorophyll b was not deficient in the chloroplasts of mesophyll cells in maize leaves and of chlorenchyma cells in barley leaves (Thornber et al., 1967; Woo ***et al.,*** 1970; Pyliotis ***et al.,*** 1971; Lee *et al.,* 1977). Therefore, the amount of chlorophyll was considered to be still higher in barley than in maize leaves.

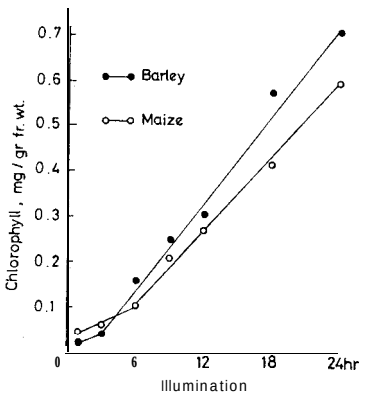


**Fig. 3.** Total chlorophyll content following the chloroplast development.

## DISCUSSION

$CO_2$ fixation rate was not so much increased until 1 hour illumination

stage in barley as well as in maize leaves, but increased afterwards as the chloroplast development. These results were very closely correlated with an increase in total chlorophyll contents and grana formation following the chloroplast development as reported in the previous paper (Lee et ***al.,*** 1977). $CO_2$ fixation was about 2 times higher in light fixation and 2-9 times higher in dark fixation in maize leaves than in barley leaves. These suggest that phosphoenolpyruvate carboxylase activity is higher even in the etiolated leaves of maize seedlings than barley seedlings and also is stimulated by light (Stabenau, 1972; Kamiya and Miyachi, 1975).

Rhodes and Yemm (1966) reported that grana formation was promoted by light and coincided with chlorophyll formation and an increase in photosynthetic activity in barley seedlings. Wieckowski (1969), using very young bean seedlings, found that $O_2$ evolution was first detectable after about 2 hours of illumination, and this corresponded with the appressed lamellae in the plastid. But grana formation is generally correlated with photosystem II, but not with $O_2$ evolution (Anderson and Boardman, 1964; Gyldenholm and Whatley, 1968; Sane et ***al.,*** 1970). And also Robertson and Laetsch (1974), using each region of a barley leaf, reported that membrane apprcssion was not strictly correlated with the presence of chlorophyll and photosynthetic activities. In addition, a barley mutant lacking chlorophyll b exhibited high photosystem I and II activities in spite of reduction of appressed lamellae (Highkin and Frenkel, 1962; Goodchild et ***al.,*** 1966). We also reported that there were many appressed lamellae even in the etioplasts (Lee ***et al.,*** 1977). In this result, chlorophyll could be detected after about 1 hour illumination. So we agreed that appressed lamellae were not strictly correlated with the presence of chlorophyll. But chlorophyll formation was stimulated by light. Both ATP and NADPH were generated as chlorophyll harvested quanta and also Hill reaction was stimulated (Gyldenholm and Whatley, 1968 ; Oelge-Karow and Butler, 1971). Such cummulative results led us to conclude that an increase of $CO_2$ fixation was closely correlated with chlorophyll formation and accumulation as the chloroplast development.

On the other hand, in maize leaves, a malate former, proportion of total $^{14}C$ incorporated into aspartate was much higher than into malate in the leaves illuminated for 3 hours. With the chloroplast development, $^{14}C$ was much incorporated into malate as in the leaves cultured in a green house. Hatch (1973) and Hatch and Mau (1973) reported that aspartate aminotransferase was about equally distributed between mesophyll and bundle sheath cells. Aspartate aminotransferase in mesophyll cells was associated with choroplasts or other subcellular organelles, but the major aspartate aminotransferase isoenzyme in bundle sheath cells was associated with mitochondria in aspartate formers such as *Atriplex spongiosa* and ***Panicum miliaceum.*** However, in maize seedlings, mitochondria were well differentiated even in the mesophyll as well as in the vascular bundle sheath cells after 3 days of dark germination, but chloroplasts were not differentiated until 3 hours illumination to etiolated leaves of 6 days dark-grown seedlings (Lee ***et al.,*** 1977). And also aspartate aminotransferase activity was not increased following the

chloroplast development (Lee *et al.*, unpublished data). Moreover, the activity of NADP malate dehydrogenase was low in etiolated leaves of maize seedlings grown at dark, but increased 10–20 folds together with chlorophyll when leaves were illuminated (Johnson and Hatch, 1970). Therefore, we considered that oxaloacetate produced by phosphoenolpyruvate carboxylase will be transformed into aspartate rather than malate by aspartate aminotransferase originated from mitochondria and other organelles except chloroplasts at an early stage of the chloroplast development, but by further development of chloroplasts oxaloacetate can be transformed into malate by increased NADP malate dehydrogenase.

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