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# CHLOROPLAST LIPID SYNTHESIS AND MITOCHONDRIAL CYTOCHROME *c* OXIDASE ACTIVITIES IN BARLEY LEAVES

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**Key Word Index**—*Hordeum vulgare*; Gramineae; barley; senescence; chloroplast lipids; cytochrome *c* oxidase.

**Abstract**—Some biochemical changes of the chloroplast lipids and of the cytochrome *c* oxidase activity of the mitochondria of barley leaf tissue of varying developmental stages, grown both in the light and in the dark, were investigated. Generally, the dark treatment reduced the incorporation of hexose into the galactolipids of the chloroplast. Isolated protoplasts were more affected than whole tissues. Also, acetate incorporation into whole leaf tissues was reduced by dark treatment. An investigation of acetate incorporation to the acyl groups of phosphatidylglycerol, a chloroplast lipid, and phosphatidylethanolamine, a non-chloroplast lipid, was made. Also, dark treatment reduced the activity of cytochrome *c* oxidase.

## INTRODUCTION

The first ultrastructural signs of senescence appear in the chloroplast [1, 2]. Plastids become closely appressed to the plasmalemma, the average plastid size decreases [3, 4] and the normal elliptical shape gives way to a more spherical one [4]. Even though the chloroplast shows early ultrastructural changes during senescence, it is the last organelle to remain in the cell [1]. On the other hand, the mitochondria show little ultrastructural change during senescence except for some swelling of cristae [4].

In order to study senescence in these organelles more completely, we decided to investigate some biochemical changes of mitochondria and chloroplasts by determining the activities of marker enzymes. Other investigators have used enzymes to study the senescence phenomenon. Lai and Thompson [5] found that the activities of two enzymes associated with the plasmalemma declined during senescence. Catalase, the marker enzyme for microbodies, has higher activity in mature leaves than in immature or senescent ones. When senescence is delayed catalase activity increases [6, 7]. Nakayama and Ashai [8] investigated the activity of succinate dehydrogenase in pea cotyledons during germination in the inner mitochondrial membrane and found increasing lability and weakness of the membranes with age.

Cytochrome *c* oxidase is a widely recognized marker enzyme for mitochondria. Galactosyl transferase is located in the chloroplast envelope and is responsible for the final assembly of mono- (MGDG) and digalactosyldiacylglycerol (DGDG), the most abundant and extremely important lipids of the chloroplast thylakoids.

The plant tissue chosen for this study was barley grown in the light for *ca* one week. Some plants were then placed in the dark to induce senescence. Of these dark-senesced plants, some were placed in the light again to induce the green control condition.

## RESULTS AND DISCUSSION

The dark treatment somewhat reduced the incorporation of hexose into MGDG of whole tissue, and this partially recovered by regreening the tissue (Table 1). However, the dark treatment markedly reduced the incorporation of hexose into the glycolipid of isolated protoplasts. Apparently an additional 6 hr incubation with the UDP-galactose, in most cases, did not affect an increased labeling of the galactolipids, a little more than for dark and regreened whole tissues but not strikingly so. Either the MGDG was further metabolized or the rate of MGDG synthesis, under these experimental conditions, was markedly decreased after 6 hr. However, both could have occurred. We would suggest from these data that, at least with respect to the galactolipid metabolism, the isolated protoplasts were not the same as whole leaf tissue. This is true, even though there may have been differential rates of penetration of the label, since the protoplasts isolated from dark-treated tissues, as compared to those isolated from tissues given only a light treatment, showed such a drastic reduction in label incorporation from UDP-[$^{14}$C]galactose. The UDP-[$^{14}$C]galactose is a specific precursor for the hexose moiety of the galactolipids since we have repeatedly found that these, and the steryl glycosides and acylated steryl glycosides, are the products which become labeled [9]. Obviously, therefore, the $^{14}$C is not found in the acyl groups of these and the other lipids. MGDG and phosphatidylglycerol (PG) are chloroplast lipids; whereas phosphatidylethanolamine (PE) is an extra-chloroplastic membrane lipid.

There seems to be a slight increase in acyl group formation in whole leaf tissue between hours 6 and 12 of labeling. There may, also, have been some turn-over of the lipids during this time; since we have shown previously that the $t_{1/2}$ of these acyl groups, under these conditions, is probably *ca* 24 hr [10]. It is apparent, therefore, that the protoplasts differ more, compared to the whole tissue, in the incorporation of label into the hexose moiety of MGDG than into the acyl groups of the same lipid, when dark-treated tissue is compared to light-treated tissue. The dark treatment also affected the acyl group synthesis of PG (another chloroplast lipid; [11]) during the 6 and 12 hr labeling periods in both whole tissue and isolated protoplasts.

The labeling of PE (a non-chloroplast, membrane lipid)

Table 1. Incorporation of hexose from UDP-[$^{14}$C]galactose or $^{14}$C from acetate into MGDG of barley whole leaf tissues or protoplasts isolated from barley leaf tissues

| | UDP-galactose incorporated (dpm) | | | | Acetate incorporated (dpm) | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | Whole tissue | % control | Protoplasts | % control | Whole tissue | % control | Protoplasts | % control |
| C-6 | 2780 | — | 10100 | — | 36800 | — | 4250 | — |
| D-6 | 1680 | 60* | 2240 | 22 | 26300 | 71 | 3640 | 86 |
| R-6 | 2410 | 87 | 3910 | 36 | 33900 | 92 | 5190 | 122 |
| C-12 | 2300 | — | 10960 | — | 41800 | — | ND† | — |
| D-12 | 3030 | 132 | 2200 | 20 | 29700 | 71 | ND | — |
| R-12 | 3420 | 149 | 3470 | 32 | 38900 | 93 | ND | — |

*Percent change of control.

†Not determined because the protoplasts did not remain intact.

Barley whole leaf tissue (1 g) was incubated in 1 $\mu$Ci UDP-[$^{14}$C]galactose or 5 $\mu$Ci [$^{14}$C]acetate in a 15 ml volume for 6 or 12 hr; or $10^9$ protoplasts isolated from barley leaf tissue were incubated in 1.67 $\mu$Ci UDP-[$^{14}$C]galactose or 5 $\mu$Ci [$^{14}$C]acetate in a 2.5 ml volume for 6 or 12 hr, all in the light. The barley was either 7-day-old (control, C), or 7-day-old barley which had been placed in the dark for 2.5 days (dark-senesced, D), or 7-day-old barley which had been placed in the dark for 2.5 days then placed in the light for an additional 2.5 days (regreened, R).

in whole tissues was decreased by the dark treatment after 12 hr but not after 6 hr of labeling (Table 2). However, the dark treatment did affect the labeling after 6 hr in protoplasts isolated from these tissues. There appeared to be continued synthesis of the acyl groups of PE between 6 and 12 hr in whole tissues which were grown in the light only or which were regreened. We found previously that senescent (nitrogen-deficient) tissues have a reduced capacity for synthesizing chloroplast lipids, more so than for the synthesis of lipids more characteristic of membranes other than those of the chloroplast [12]. This seems to be true in these studies also, except for longer labeling times. Also, protoplasts isolated from dark-treated tissue incorporated less acetate label than did the protoplasts from control tissue during the 6 hr labeling period.

Generally, therefore, the kinetics of lipid metabolism of protoplasts is not the same as that of whole tissues. In addition, penetration problems aside, the labeling of the hexose moiety of MGDG was not similarly affected as that of the acyl groups in this same lipid by the dark treatment. Isolated protoplasts are not the same in every aspect of their metabolism as the cells of whole tissues. Wittenbach *et al.* [13] found that protoplasts isolated from wheat leaves during senescence first showed a decline in photosynthesis, then a decline in ribulose bisphosphate carboxylase activity and finally a decline in chloroplast number. A comparison cannot be made between the rates of incorporation by the unknown number of protoplasts in segments of whole tissues with that of the known number of isolated protoplasts. What is evident, however, is the greater rate of hexose incorporation into the glycolipids of isolated protoplasts (when compared to the intact segments) as compared to the rate of acyl group synthesis of the glycolipid in isolated protoplasts (again as compared to intact segments). Haas *et al.* [14] estimate that when protoplasts of oat leaves are made, some $10^3$–$10^4$ protoplasmic connections between neighboring cells, per protoplast formed, are cut. One

Table 2. Incorporation of $^{14}$C from acetate into PG and PE of barley whole leaf tissues or protoplasts isolated from barley leaf tissues

| | Label incorporated into PG (dpm) | | | | Label incorporated into PE (dpm) | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | Whole tissue | % control | Protoplasts | % control | Whole tissue | % control | Protoplasts | % control |
| C-6 | 23400 | — | 6100 | — | 31300 | — | 4900 | — |
| D-6 | 13900 | 59* | 2600 | 43 | 30000 | 96 | 1200 | 24 |
| R-6 | 16400 | 70 | ND† | — | 21700 | 69 | ND | — |
| C-12 | 31900 | — | ND | — | 57500 | — | ND | — |
| D-12 | 14000 | 44 | ND | — | 28200 | 49 | ND | — |
| R-12 | 16300 | 51 | ND | — | 39500 | 69 | ND | — |

*Percent change of control.

†Not determined because the protoplasts did not remain intact.

Barley whole leaf tissue was incubated in 5 $\mu$Ci [$^{14}$C]acetate in a 15 ml volume for 6 or 12 hr; or $10^9$ protoplasts isolated from barley leaf tissue were incubated in 5 $\mu$Ci [$^{14}$C]acetate in a 2.5 ml volume for 6 or 12 hr, all in the light. The barley was either 7-day-old (control, C), or 7-day-old barley which had been placed in the dark for 2.5 days (dark-senesced, D), or 7-day-old barley which had been placed in the dark for 2.5 days then placed in the light for an additional 2.5 days (regreened, R).

wonders about the rates of transport of label, across the plasmalemma of intact cells, as compared to that of isolated protoplasts and what effect this may have on the rates of hexose incorporation, from UDP-hexose and of acyl group synthesis from acetate, in the tissues of varying developmental stages. This, in addition to the activity of metabolic synthesis of these compounds, must be considered [15]. Haas and Heinz [11] have suggested that some aspects of lipid metabolism in isolated protoplasts are different from those of intact tissues. Indeed they found rather low rates of penetration of nucleoside diphosphate hexose through the plasmalemma. Others have not found this to be so [16].

Generally speaking, cytochrome *c* oxidase activity decreased in dark-senesced tissues indicating that the mitochondria are affected during senescence (Tables 3 and 4). Also, tissues of varying ages which have been grown in the same light regime show differing cytochrome *c* oxidase activities. In most cases the tip of the leaf had a higher activity than the next 1 cm segment down from the tip of the same leaf, which in turn had a lower activity than did the segment which was *ca* 5 cm down from the tip (even younger tissues). In general, it appears that the measured cytochrome *c* oxidase activity is sensitive to the developmental stage of the tissue; mitochondria may indeed be affected by senescence.

## EXPERIMENTAL

*Isotope incubation.* 1 g barley (*Hordeum vulgare* L. grown at 9.5 klx) leaf tissue was incubated in a covered Petri dish containing 15 ml $H_2O$ and either 1 $\mu$Ci UDP-[$^{14}$C]galactose (UDP-[$^{14}$C]gal) or 5 $\mu$Ci [$^{14}$C]acetate. Incubation times were for 6 or 12 hr. Protoplasts were incubated as indicated in Tables 1 and 2.

*Protoplast isolation.* Expanded blades were rubbed with carborundum, rinsed and surface sterilized. The protoplast isolation medium consisted of 25 mM KPi–citrate buffer (pH 5.5), 0.5 M mannitol and 0.75% (w/v) Onozuka cellulase. The expanded blades were floated on 25 ml of medium for 3 hr at 37° on a gyratory shaker.

*Lipid extraction.* Before extraction, leaves were steam-killed for 30 min to prevent the generation of phospholipid artifacts during the extraction process [17]. The lipids were extracted according to ref. [18].

*Lipid identification.* The lipids were separated by TLC on Si gel G ($CHCl_3$–MeOH–7 M $NH_4OH$, 65:25:4 in the first dimension and $CHCl_3$–MeOH–HOAc–$H_2O$, 170:15:15:2 in the second dimension). Phospholipids were identified by the method of ref. [19] and glycolipids by the method of ref. [20]. The labeled lipids were counted by liquid scintillation spectrometry using 2,5-bis-2-(5-tert-butylbenzoxazolyl)-thiophene (BBOT) as the fluor and were then recounted using an internal standard of [$^{14}$C]palmitic acid.

*Cytochrome c oxidase.* Leaf tissue (0.3 g) was ground in 10 ml 0.05 M Pi buffer, pH 7.5, containing 0.3 g PVP. The extract was filtered through Miracloth and centrifuged at 600 *g* for 3 min. Digitonin (100 $\mu$l) was then mixed with 200 $\mu$l of supernatant. To 100 $\mu$l of this soln was added 800 $\mu$l 0.05 M KPi buffer, 100 $\mu$l cytochrome *c* and the *A* measured at 550 nm.

Table 3. Cytochrome *c* oxidase activities of barley leaf tissues of varying developmental stages

| | Cytochrome *c* oxidase activity (n mol/min·g fr. wt) | | |
|---|---|---|---|
| Light treatment | Top 2 cm of leaf | Second 2 cm from leaf tip | Third 2 cm from leaf tip |
| 8 days light | 1890 ± 300 | 1710 ± 130 | 1900 + 170 |
| 8 days light; 2 days dark | 1560 ± 70 | 1520 ± 160 | 1610 ± 30 |
| 14 days light | 2850—* | 2510— | 2300 ± 352 |
| 8 days light; 2 days dark; 4 days light | 2540 ± 220 | 1890 ± 310 | 2580 ± 220 |

*Only two samples.

Table 4. Chlorophyll contents of barley leaf tissues of varying developmental stages

| | Chlorophyll content (mg chl/g fr. wt) | | |
|---|---|---|---|
| Light treatment | Top 2 cm of leaf | Second 2 cm from leaf tip | Third 2 cm from leaf tip |
| 8 days light | 1.2* | 1.5 | 1.3 |
| | 0.51† | 0.73 | 0.53 |
| 8 days light; 2 days dark | 1.3 | 1.1 | 1.3 |
| | 0.85 | 0.72 | 1.1 |
| 14 days light | 1.6 | 1.6 | 1.2 |
| | 1.2 | 1.2 | 1.1 |
| 8 days light; 2 days dark; 4 days light | 1.4 | 1.2 | 1.4 |
| | 0.93 | 0.91 | 1.2 |

*Chlorophyll *a*.
†Chlorophyll *b*.

## REFERENCES


1. Dodge, J. D. (1970) *Ann. Botany* **34**, 817.
2. Mlodzianowski, F. and Ponitka, A. (1973) *Z. Pflanzenphysiol.* **69**, 13.
3. Ljubesic, N. (1968) *Protoplasma* **66**, 369.
4. Barton, R. (1966) *Planta* **71**, 314.
5. Lai, Y. F. and Thompson, J. E. (1972) *Plant Physiol.* **50**, 452.
6. Patra, H. K., Kar, M. and Mishra, D. (1978) *Biochem. Physiol. Pflanzen.* **172**, 385.
7. Barber, J. M. (1970) *Z. Pflanzenphysiol.* **97**, 135.
8. Nakayama, N. and Ashai, T. (1981) *Plant Cell Physiol.* **22**, 79.
9. Eichenberger, W. and Newman, D. W. (1968) *Biochem. Biophys. Res. Commun.* **32**, 366.
10. Hanna, K. S. and Newman, D. W. (1975) *Phytochemistry* **14**, 1293.
11. Haas, R. and Heinz, E. (1980) in *Biogenesis and Function of Plant Lipids* (Mazliak, P., Benveniste, P., Costes, C. and Douce, R., eds.) pp. 19–28. Elsevier-North Holland Biomedical Press, Amsterdam.
12. Dalgarn, D., Huber, D. and Newman, D. W. (1977) *Physiol. Plant.* **40**, 153.
13. Wittenbach, V. A., Lin, W. and Hebert, R. R. (1982) *Plant Physiol.* **69**, 98.
14. Haas, R., Heinz, E., Popovici, G. and Weissenbock, G. (1979) *Z. Naturforsch.* **34**, 854.
15. Kuhn, D. N., Knauf, M. and Stumpf, P. K. (1981) *Arch. Biochem. Biophys.* **209**, 441.
16. Rathnam, C. K. M. (1978) *Plant Physiol.* **51**, 229.
17. Roughan, P. G., Slack, C. R. and Holland, R. (1978) *Lipids* **13**, 497.
18. Dalgarn, D. S. and Newman, D. W. (1979) *J. Exp. Botany* **30**, 551.
19. Dittmer, J. C. and Lester, R. L. (1964) *J. Lipid Res.* **5**, 126.
20. Siakotos, A. N. and Rouser, G. (1965) *J. Am. Oil Chem. Soc.* **42**, 913.