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MODIFICATIONS OF THYLAKOID LIPIDS IN *EUGLENA GRACILIS* DURING DIURON-ADAPTATION

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Key Word Index—*Euglena gracilis*; Euglenophyceae; algae; diuron-resistance; role of galactolipids in photosynthesis; fatty acid metabolism.

Abstract—The adaptation of *Euglena gracilis* to 25 μM diuron leading to a new resistant strain ZR results from a dedifferentiation then a redifferentiation of the photosynthetic structures and activities. The interactions between lipids and photosynthetic ability, and the biosynthetic pathways of galactolipid fatty acids were studied by following lipid changes (classes and fatty acids). During adaptation strong correlations existed between monogalactosyl diacylglycerol, digalactosyl diacylglycerol and chlorophyll for photochemistry. During the first weeks of treatment, diuron seemed to inhibit fatty acid desaturation and activated elongation. It was concluded that the mutation of the M_r 32–34 K protein produced by the diuron action is accompanied by lipid changes of the thylakoid matrix.

INTRODUCTION

Diuron [3-(3,4 dichlorophenyl)-1,1 dimethyl urea; DCMU] is generally used as a photosynthesis inhibitor. At low concentrations (10^{-8} M) it blocks the electron transfer between Q (the primary electron acceptor of photosystem II) and the plastoquinone pool [1]. Cyclic photophosphorylation [2] and respiratory electron transfer [3] could be inhibited by higher concentrations (10^{-4} M). High concentrations affected the growth rate of *Escherichia coli* [4] or *Eudorina* [5] in aerobic or anaerobic conditions of culture. Diuron also depressed chlorophyll synthesis [6–8], as well as galactolipid and fatty acid metabolisms [9, 10].

When *Euglena gracilis* Z was reinoculated weekly on fresh medium containing 33 mM DL-lactate as sole C source and 25 μM diuron, it progressively adapted to the herbicide in 15 weeks [11, 12]. This diuron adaptation was a dynamic process presenting three successive and distinct phases: (i) an initial phase characterized by a drastic drop in chlorophyll, a structural disorganization of the plastids (with unstacking and swelling of the thylakoids) and loss of photosynthetic capacity [12]; (ii) a second phase during which progressive regreening occurred which led transitorily to 'super algae' with better photosynthetic performances than the control [12]; (iii) a last phase which corresponded to diuron-resistant ZR strain exhibiting stable changes at the level of diuron sensitive sites [13–15] and metabolic properties [16]. Recent work on this ZR strain has shown a mutation on the M_r 32 k protein [17].

We present here a detailed study of lipid changes during such an adaptation to diuron and especially emphasize the galactolipids specific to the thylakoids. Moreover we attempt to correlate these changes with the structural and functional alterations that we had previously analysed [11, 12].

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RESULTS

Reproductibility of the results

On analysis of the control Z cells in triplicate, accuracy was ±5% for the lipids and ±8% for the fatty acid compositions.

Purity of the thylakoid fractions

The glycoglycerolipids [monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and sulphoquinovosyl diacylglycerol (SQD)] are specific to thylakoids, and phospholipids (PL) can provide a measure of contaminating, non-thylakoid membranes. Our data from *Euglena* were thus compared in Fig. 1 with data published for barley [18] and *Dunaliella salina* [19]. The striking high proportion (62%) of PL characterizing the control Z strain in comparison with the *ca* 20% found in higher plants could suggest an important contamination by microsomal membranes. However, we think that the contamination is much less than it seems, since Lynch and Thompson [19] claimed a contamination less than 7%, but found 43% of associated PL in fractions from *Dunaliella* highly enriched in chloroplasts and examined by electron microscopy. Moreover we show in subsequent results (Fig. 2) that during adaptation, this PL proportion falls to a 22% minimum to reach 36% in ZR.

Changes in membrane components during adaptation

We have studied the compositions of total lipids, proteins and chlorophyll (Chl) in the thylakoid fractions.

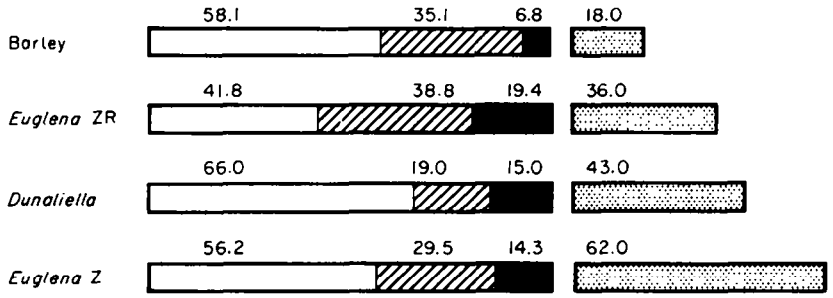


Fig. 1. Distribution in chloroplast fractions of the three different glycoglycerolipids (MGDG, □; DGDG, ▨ and SQD, ■) as percent of their sum and representation of the amounts of associated phospholipids (PL), ▩. Data concerning *Euglena gracilis* control Z strain and diuron adapted ZR strain are compared with data for barley [18] and *Dunaliella salina* [19].

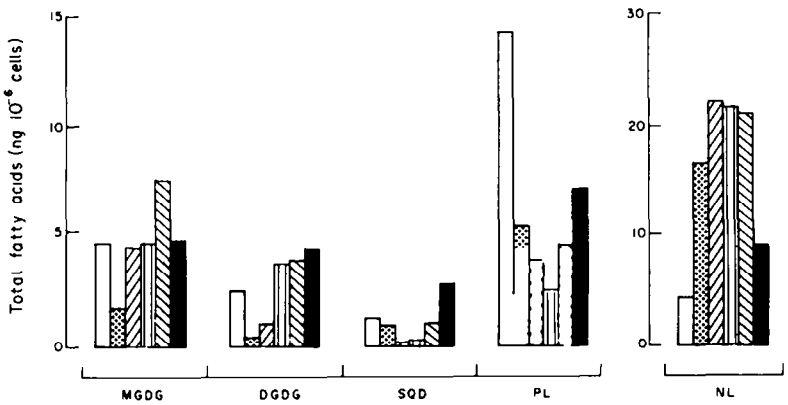


Fig. 2. Total fatty acids among the different lipid classes during the adaption to 25 μM diuron of *Euglena gracilis*. Control Z strain, □; at 3–4 weeks of treatment, ▩; at 6–7 weeks, ▨; at 7–8 weeks, ▥; at 8–9 weeks, ▧ and when totally adapted they becarne ZR strain, ■.

Table 1. Contents of thylakoid-enriched fractions during adaptation of *Euglena* to 25 μM diuron

	Control	during adaptation (weeks on diuron)				adapted
	Z	3–4	6–7	7–8	8–9	ZR (15)
			(μg per 10^6 cells)			
Lipids	28.2	25.6	32.0	33.4	38.9	29.1
Proteins	79.4	81.2	186.1	130.3	139.7	144.2
Chl	7.8	1.2	4.8	8.4	10.0	8.0

The results (Table 1) showed that the lipids never varied by more than 40%, while proteins could increase by 135% and Chl decrease by 85% during the adaptation process. At 3–4 weeks, while the bleaching of membranes occurred, simultaneous changes affected lipids and Chl (the Chl almost disappeared from a lipid matrix quantitatively only weakly modified) when the total protein seemed stable. In subsequent stages the lipids and particularly the proteins then accumulated as soon as the new synthesis of Chl took place (6–7 weeks).

The glycoglycerolipids of the chloroplasts from Euglena gracilis *Z*

The richness of *Euglena* in MGDG was close to that of barley, while the DGDG content was intermediate between those of algae and the higher plants (Fig. 1). An abundant SQD pool seemed to be characteristic of algae (Fig. 1); nevertheless, differences in the fatty acid compositions of this pool appeared between *Euglena* and *Dunaliella* (Table 2), the former exhibiting the highest

Table 2. Fatty acid composition, as percent of total fatty acids, of the glycoglycerolipids in barley [18], *Euglena gracilis* Z (values expressed as mean of three separate experiments) and *Dunaliella salina* [19]

	Fatty acids (% of total acids)									
	16:0	16:1	16:2	16:3	16:4	18:1	18:2	18:3	20:3	22:1
MGDG										
Barley	4.1	1.0	—	—	—	1.0	3.0	90.0	—	—
Euglena	1.0	2.4	5.8	11.2	20.0	—	8.0	48.8	—	—
Dunaliella	0.3±0.1	—	0.6±0.1	2.8±0.6	47.8±0.7	—	2.8±0.7	45.8±1.3	—	—
DGDG										
Barley	9.0	2.0	—	—	—	3.0	7.0	78.0	—	—
Euglena	3.5	2.0	7.2	27.8	6.0	—	6.8	44.0	—	—
Dunaliella	29.3±0.6	—	5.0±0.6	12.3±1.4	4.0±0.3	—	9.5±1.5	38.6±1.4	—	—
SQD										
Barley	32.0	3.0	—	—	—	2.0	5.0	55.0	—	—
Euglena	23.6	—	1.4	—	—	17.4	0.5	1.4	24.5	12.1
Dunaliella	61.8±2.2	—	—	—	—	3.4±0.8	4.5±0.3	22.6±0.6	—	—

degree of unsaturation and a very high content in C20:3 in place of C18:3. The SQD from barley presented a fatty acid composition intermediate between those of *Euglena* and *Dunaliella*. Conversely, the fatty acid compositions of both MGDG and DGDG from *Euglena* (Table 2) were closer to the ones of *Dunaliella* than to the ones of barley, because of the presence of high concentrations of C16:3 and C16:4 at the expense of C18:3.

Lipid changes during diuron-adaptation

During the 15 weeks leading from control Z strain to the complete adaptation to 25 μM diuron of ZR strain, we have followed both changes in the lipid classes and the modifications affecting the fatty acid compositions of each of the categories.

Time-course of the lipid class changes. During diuron-adaptation, major changes occurred among the thylakoid lipid classes (Fig. 2). There were antiparallel alterations of PL and of neutral lipids (NL). Simultaneously the glycoglycerolipids (MGDG, DGDG and SQD) exhibited comparable changes: there was a concomitant 70% decrease in both galactolipids and PL at 3 weeks when thylakoidal destacking began [11] and cellular Chl content decreased (Table 1); SQD dropped slightly later when regreening began (stage 6–7 weeks). During the first 4 weeks of treatment, a strong enrichment of cells in NL occurred which persisted even when photosynthetic characteristics reappeared (Table 1 and ref. [12]).

In *Euglena*, diuron should have a comparable inhibitory effect on biosynthesis of PL and GL from phosphatidic acid (PA) which had been already described in *Chlorella* [9]. When photosynthetic characteristics were restored (sixth week), the MGDG pool returned to the control value while the DGDG pool remained low and the SQDs were minimal. Later on, DGDG and SQD increased when NL decreased.

When cells were totally diuron-adapted the MGDG contents were identical in Z and ZR cells. Moreover the new ZR strain could be characterized by reference to the control Z strain by: 200% of NL, 170% of DGDG and SQD but only 40% in PL. Despite this redistribution of lipid classes between Z and ZR, photosynthetic O_2 evolution was identical [12, 14].

Fatty acid distribution among the different lipid classes during the diuron-adaptation. The data expressed as percent of the total fatty acids are presented in Table 3. We selected the ten principal species among the nineteen detected, neglecting the C14:1, C14:2, C16:2, C18:0, C18:2, C18:4 and C20:4 whose sum represented only *ca* 20% of the total. For a given class of lipids, there was a relative stability of the sum C14:0, C16:0, C16:1, C16:3, C16:4, C18:1, C18:3, C20:2, C20:3 and C22:1-2 during the diuron-adaptation.

The fatty acids from galactolipids (GL) were present only as C_{16} or C_{18} while the length of the C-chain of those from PL and NL varied from 14 to 22. The GLs were almost devoid of saturated fatty acids and contained most of the polyunsaturated C_{16}–C_{18} acyl chains having three or four double bonds. The SQD, as the GL, contained only C16:0 as saturated fatty acid; the double bonds are only found in acyl chains having more than 16 carbon atoms. The PL and NL, which seemed to be the storage molecules for C14:0 and C16:0, also contained very long (up to C_{22}) polyunsaturated acyl chains.

We then analysed the results of Table 3 by following the redistribution of given acyl chains in a given class of lipid as a function of the three developmental phases leading to ZR.

Phase (i), loss of photosynthetic ability: the C14:0 strongly increased in PL and NL; the C16:0 which diminished in NL stayed relatively stable in PL and slightly increased in GL; the C16:4 disappeared from all classes; in MGDG, where this C16:4 was the most abundant, there was a simultaneous accumulation of less unsaturated C16-acyl chains. A large increase of C18:1 and C20:3 fatty acids, absent from the Z strain, in the DGDG compensated for the drastic drop of both C16:3 and C18:3, as well as the total disappearance of C16:4. Moreover C20:3 and C20:2 increased in both NL and PL, classes in which C22:1-2 dropped.

Phase (ii), progressive regreening leading to high efficiency in photosynthesis, was very complex (Table 3). Nevertheless the fatty acids tended globally to stabilize to a distribution close to the one in the control Z strain.

Phase (iii): new changes in lipids occurred when the diuron-treatment was extended for 6 more weeks and led to the acquirement of a permanent resistance to the

Table 3. Fatty acid distribution in the different lipid classes during the adaptation of *Euglena gracilis* to 25 μM diuron

	14:0	16:0	16:1	16:3	16:4	18:1	18:3	20:2	20:3	22:1-2	Total
MGDG											
Z strain	—	1.0	2.4	11.2	20.0	—	48.8	—	—	—	83.4%
3–4 weeks	—	5.7	9.0	22.3	—	—	44.3	—	—	—	83.7%
6–7 weeks	—	6.0	11.1	21.1	9.8	—	39.7	—	—	—	87.7%
7–8 weeks	—	3.1	7.5	18.7	6.0	—	52.0	—	—	—	90.1%
8–9 weeks	—	2.9	6.1	10.5	11.9	—	42.8	—	—	—	81.4%
ZR strain	—	3.0	5.5	19.2	14.4	—	42.5	—	—	—	84.6%
DGDG											
Z strain	—	3.5	2.0	27.8	6.0	—	44.0	—	—	—	83.3%
3–4 weeks	—	7.6	0.6	3.2	—	9.5	23.4	—	46.8	—	91.1%
6–7 weeks	—	11.0	6.5	28.2	1.2	—	38.3	—	—	—	85.2%
7–8 weeks	—	7.1	4.8	28.4	3.6	—	44.5	—	—	—	88.7%
8–9 weeks	—	5.6	3.5	24.7	5.8	—	40.7	—	—	—	80.3%
ZR strain	—	5.0	3.0	25.0	4.0	—	47.0	—	—	—	84.0%
SQD											
Z strain	—	23.6	—	—	—	17.4	1.4	—	24.5	12.1	79.0%
3–4 weeks	—	21.6	—	—	—	40.7	2.4	—	8.9	—	73.6%
6–7 weeks	—	20.4	—	—	—	14.4	7.1	—	27.1	—	69.0%
8–9 weeks	—	11.1	—	—	—	5.7	2.2	—	47.3	—	66.3%
ZR strain	—	9.6	—	—	—	2.4	4.4	—	27.8	15.9	60.0%
PL											
Z strain	7.4	17.8	5.5	3.8	1.3	3.1	21.2	2.4	5.3	13.1	71.8%
3–4 weeks	14.7	22.8	5.2	—	—	4.0	12.7	12.5	7.6	1.3	80.8%
6–7 weeks	3.4	24.2	4.5	4.2	—	7.8	19.0	—	1.5	4.7	69.3%
7–8 weeks	4.3	35.5	2.9	1.4	0.4	8.3	26.9	—	6.9	1.4	89.0%
8–9 weeks	2.2	32.5	1.9	3.1	0.7	12.4	25.2	—	5.6	2.0	85.6%
ZR strain	9.5	20.0	5.1	1.4	0.4	4.5	13.6	4.1	12.1	7.6	78.3%
NL											
Z strain	23.6	17.6	4.0	1.1	3.4	2.7	12.9	1.9	3.7	5.1	77.0%
3–4 weeks	32.2	10.8	3.6	3.0	0.1	2.7	7.1	6.5	13.5	3.9	83.3%
6–7 weeks	13.1	9.3	4.1	3.4	2.5	4.1	11.9	11.6	15.2	6.9	82.1%
7–8 weeks	10.7	11.1	3.5	2.7	2.6	6.3	17.8	1.7	10.7	8.3	75.4%
8–9 weeks	11.9	11.9	4.1	2.9	5.4	5.6	11.1	2.3	10.0	9.4	74.6%
ZR strain	28.4	7.3	5.2	1.6	2.3	3.1	6.6	2.2	9.4	18.4	84.5%

—denotes undetectable quantity (< 0.1).

herbicide. Thus the ZR resistant thylakoids were enriched in C16:3 of the MGDG, C14:0, C20:2 and C20:3 in the PL, in C14:0, C20:2, C20:3 and C22:1–2 in the NL. Conversely, there were decreases in the C16:4 of the GL; the C16:0 and C18:3 of the NL; C18:3 and C22:1–2 of the PL.

DISCUSSION

We thought that the progressive adaptation of *Euglena gracilis* to 25 μM of diuron, which occurred in 15 weeks [11, 12], could be considered as a physiological model, permitting both better understanding of the specific role of the thylakoid lipids in photosynthetic activities, and their possible interactions with the light harvesting pigment–protein complexes (LHPPC), together with the possibility of predicting a biosynthetic scheme of the GL fatty acids in *Euglena*. The adaptation process could be divided into three successive and distinct phases characterized by very different photosynthetic abilities.

The first one, accompanied by an important disappearance of Chl (Table 1) and an inhibition of photosynthetic O_2 evolution [12], corresponded to thylakoid lamellae still present (though vesiculated) and to relatively unchanged lipid and protein contents (Table 1). During this phase there was a strong enrichment in NL at the expense of the polar lipid content (Fig. 2). Furthermore, the fatty acid compositions of the different lipid classes were strongly affected. The most important change was the disappearance of the C16:4 accompanied by an accumulation of C16:3 in the MGDG but by a decrease of both C16:3 and C18:3 in the DGDG (Table 3). By way of compensation C20:3 drastically increased, suggesting the appearance of a new transitory elongation process. Such alterations in both GL and in fatty acids, in the presence of 25 μM of diuron, was peculiar to the dedifferentiation of the structures and loss of the activities in chloroplasts. This first phase could not be compared to the differentiation of chloroplasts from etioplasts during which time the lipid matrix changed only quantitatively, but not qualitatively [20].

The second phase was characterized by a two fold increase in photosynthetic activity expressed on a Chl basis compared to the Z strain [12]. There was a remarkable synthesis of Chl and restacking of the thylakoids [11]. This situation differed from the greening of dark grown *Euglena* in the presence of 10 μM diuron,

during which Chl was shown to be synthesized but photosynthesis was inefficient [21]. The cellular contents in GL increased towards the control values with a transitory 170% enrichment in MGDG (Fig. 2). There was a strong increase in C16:4 fatty acids with a concomitant disappearance of C20:3 while photosynthetic O_2 evolution began increasing [12]. Low contents in short and long acyl chains of PL seemed also to be specific to this regreening phenomenon and high contents in NL could be noticed for each of the intermediate steps analysed during adaptation to diuron.

When the third phase was reached, the lipid characteristics of the 25 μM-diuron adapted ZR cells, also able to resist atrazine [15], were: an increase in DGDG and SQD contents by 70%; identical amounts of MGDG; and PL depressed by 70%. Atrazine resistant weed species were, on the contrary, deficient in DGDG and richer in MGDG and PE than sensitive weeds [22]. Furthermore, the GL of *Euglena* were impoverished in C16:4 while the MGDG was enriched in C16:3. Such differences did not affect photosynthetic O_2 evolution. They could perhaps be correlated with the highest ratio of active to inactive Chl for the gathering of energy for photosystem II detected in ZR strain (data not shown) and/or the ability for this latter strain to present a blockage of the photosynthetic abilities in the presence of overdoses of diuron [14].

Correlations between lipids and photosynthesis

The photosynthetic activities in *Euglena gracilis* should therefore be correlated with the presence of C16:4 in GL; a MGDG content higher than the 1.8 $\mu g/10^6$ cells found in the 3 week-stage; and a C18:3 of more than 50% of the total fatty acids in DGDG. It then seemed that the GL could play a significant role in photosynthetic O_2 evolution by their richness in polyunsaturated fatty acids which could be involved in electron transport as it was already suggested by both Erwin and Bloch [23] and Chang and Lundin [24]. In addition, many experiments favoured a tight linkage between the presence of GL and PS I activity [25–27]. Conversely other authors concluded that there was a lack of evidence for a linkage between the GL and electron transport [28, 29].

We have attempted to establish possible quantitative relations between: the photosynthetic abilities [12] at the different adaptation stages, expressed as percent of the maximal photosynthetic evolution displayed by the control Z cells which are completely photosynthetically active; and the linkage between the various lipid classes (Table 4). We have therefore calculated indices of such a linkage as follows:

$$I_l = 100 \times \frac{(\text{lipid class})t \times (\text{Chl})t}{(\text{lipid class})z \times (\text{Chl})z}$$

where: I_l is the linkage index; (lipid class)t represents the cellular content of a given lipid class for the adaptation stage t (t being expressed in weeks); (lipid class)z represents the cellular content of the *same lipid class* for the control, Z strain (i.e. $t = 0$ week); (Chl)t is the cellular content in Chl for stage t; (Chl)z is the cellular content in Chl for Z strain.

The linkage indices were then equal to 100 for all lipid classes in control Z cells, also characterized by 100% O_2 evolution.

The results of this mathematical exercise have shown that the MGDG linkage indices were remarkably close to the photosynthetic O_2 evolution for each of the diuron adaptation stages (Table 4). The DGDG linkage indices varied in parallel but always stayed higher than photosynthetic O_2 evolution. The linkage indices for the two other lipid species drastically diverged from the photosynthetic activities, suggesting that neither PL nor NL play a major role in photosynthetic electron transfer. Our results strongly suggest the linkage between GL and photoactive Chl which had been proposed earlier [23–27]. We conclude that a very tight linkage exists between Chl, photosynthesis and MGDG. Our results reinforce the hypothesis that DGDG could stabilize protein conformation in ZR thylakoids [30], which contain 180% more membrane proteins than the control cells (Table 1). Here the linkage indices remained very high.

Biosynthesis of polyunsaturated fatty acids in GL

The conversion from oleate to linoleic acid then linolenate are well established in different photosynthetic [31–33] as well as nonphotosynthetic [34, 35] tissues. This metabolic chain is thought to reflect cooperative chemical reactions between the microsomal and the chloroplastic compartments [35].

Three to four weeks after diuron-treatment, because of the lack of photosynthesis, we anticipated a great increase in saturated GL. Instead the GL pools drastically decreased as these lipid classes were linked to photosynthetic activities because of the high degrees of unsaturation of their fatty acids. We thus observed a redistribution of the fatty acids with three or four double bonds. During the disorganization phase the disappearance of C16:4 from both MGDG and DGDG (Table 3) indicated that the last

Table 4. Evolution, during the adaptation of *Euglena gracilis* to 25 μM diuron, of the linkage indices (as defined in the text) for the different lipid categories

	Z	3–4 weeks	6–7 weeks	7–8 weeks	8–9 weeks	ZR
MGDG	100.0	5.7	58.5	105.4	204.0	102.3
DGDG	100.0	2.4	25.9	160.6	202.5	180.1
PL	100.0	3.9	14.9	17.8	24.4	36.8
NL	100.0	60.0	319.6	547.9	634.1	222.9
O_2 evolved	100%	0%	56%	125%	175%	100%

The photosynthetic O_2 evolutions, expressed as percent of the control, are already published [12].

of the complex reactions of desaturation leading from C16:0 to C16:4 was completely inhibited.

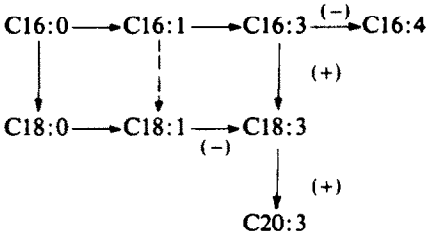
The accumulation in DGDG (Table 3) of C18:1, the intermediate molecule of the C18 series suggested two possible pathways for the biosynthesis of polyunsaturated fatty acids in *Euglena gracilis*. The first pathway could consist, as previously described [31–33], in a desaturation process from oleic acid to linoleate. The second one could lead to a $\Delta^{9,12,15}$ octadecatrienoic acid by elongation of a $\Delta^{7,10,13}$ hexadecatrienoate [35]. When the fatty acid distribution was affected by the diuron action (3–4 weeks), these two processes of desaturation and elongation seem to be modified since: (i) an additional elongation led from C18:3 to C20:3; (ii) the increase in C18:1 in the DGDG indicated that the desaturation pathway leading to C18:3 was depressed.

In future experiments we could therefore test the following simplified model of the fatty acid synthesis in GL of *Euglena*:

for MGDG:

$$C16{:}0 \longrightarrow C16{:}1 \longrightarrow C16{:}3 \xrightarrow{(-)} C16{:}4$$

for DGDG:



[effects of diuron during the first four weeks of treatment are indicated as (−) for inhibition and (+) for activation].

We have shown in this paper that diuron-resistance, which results from a mutation in the chloroplast gene for the M_r 32 K protein of PS II in both *Chlamydomonas* [36] and *Euglena* [17] is also accompanied by lipid changes. These changes, which affected the cellular contents in the different lipid classes more than the compositions in fatty acids of each of these classes, should result in differential organization of protein–pigment domains in the thylakoids [37]. It has been shown recently [38] that cerulenin treated *Chlorella*, whose lipid composition was modified, displayed diuron-resistance.

The process of adaptation characterized by deeper lipid changes provides a dynamic model for future studies of both the relations between the photosynthetic abilities, the lipid–protein–pigment functional organization and the pathways of GL biosynthesis (and associated fatty acids).

EXPERIMENTAL

Cells and cultures. Euglena gracilis Klebs strain Z Pringsheim were grown at pH 3.5 in liquid medium containing 33 mM DL-lactate as the sole carbon source [39] and 25 μM diuron added in *iso*-PrOH (2 ml per l.). The fresh cultures were inoculated weekly at 2×10^4 cells per ml of medium from the previous ones. Growth occurred at 27°, in 800 ml of medium placed in 20 cm diam. 2 l. flasks, under constant illumination of 2500 lux provided by daylight fluorescent tubes (Philips). The cell population being asynchronous there is no risk of rhythmic diurnal lipid changes [40].

Thylakoid extraction. Algae were sampled in their transition phase of growth, i.e when lactate was exhausted and the photosynthetic activity was maximal. Thylakoids were then extracted by differential centrifugation.

Euglena were pelleted by centrifugation at 165 *g* for 10 min, rinsed in H_2O and again centrifuged. The pellet was then suspended at 1:4 (wt/vol) in an extraction buffer containing 20 mM Tris–HCl, 250 mM sorbitol, 200 mM $MgCl_2$, pH 7.6 and transferred to a 'Braun Broyeur' with 2.5 g of 2 mm glass beads/ml of cell suspension. The cell breakage was done at maximal speed 3 × 10 sec CO_2 bursts separated by 20 sec to prevent freezing. The resulting suspension was filtered through three layers of cheesecloth and centrifuged for 10 min at 300 *g*. The supernatant and the viscous green layer covering the pellet containing the chloroplastic membranes were mixed and centrifuged for 10 min at 1100 *g*. The pellet was suspended in extraction buffer, and recentrifuged for 10 min at 1100 *g*, resuspended in 20 mM Tris, 250 mM sorbitol, pH 7.5 and frozen at −20° for subsequent measurements.

Chlorophyll assay. Pigments were extracted with Me_2CO–H_2O (9:1) and spectrophotometrically determined by a kinetic method of controlled pheophytinization [41–43].

Membrane protein measurement. Prior to the determination of protein [44], the membrane proteins were solubilized for 1 hr in 2 M NaOH at 37°.

Lipid analysis. (i) *Lipid extraction from thylakoid-enriched fractions.* Thylakoid suspension (1 ml) was fixed for 15 min in 17 ml of H_2O to which 1.5 ml of 1% NaCl were added. Lipids were then extracted with 100 ml of MeOH–$CHCl_3$ (2:1). After centrifugation for 15 min at 500 *g*, the $CHCl_3$ phase was evaporated under N_2 to a final vol. of 1–2 ml. Separation of the different lipid classes and analysis of the total fatty acids was then carried out on aliquots.

(ii) *Separation of lipid classes.* A band of $CHCl_3$ soln of lipids was deposited on silica gel TCL plates (Kieselgel 60, Merck). The separation was performed in a satd atmosphere of the chromatographic solvent $CHCl_3$–Me_2CO–MeOH–HOAc–H_2O (10:4:2:2:1) [45]. After spraying the plate with rhodamine (120 mg/l.), the lipid classes were characterized under UV light and the corresponding areas of silica gel were scraped off the plate.

(iii) *Esterification and methylation of fatty acids.* 4 ml of 2% NaOH in MeOH were added to each of the tubes containing either the extract of total lipids or the silica gel impregnated with a given lipid class. The tubes were maintained for 15 min at 65°. After cooling to room temp., 2 ml of BF_3 in abs MeOH (BF3, Applied Sciences) and a known quantity of C21:0 (heneicosanoic acid, Sigma) was added as an int. standard for quantitative analyses. Tubes were heated again for 15 min at 65°. The resulting Me esters were then removed from the upper phase after addition of 10 ml pentane and 2 ml H_2O, and were dried under N_2. The Me esters were dissolved in 200 μl $CHCl_3$ and injected for separation by GC (Varian 1400) on a 3 m × 3.1 mm column (Chromosorb 80-100 mesh) packed with 4% diethylene glycol sulphonate, using N_2 as carrier gas, at 20 ml/min, at 165° isothermal using FID.

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