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LIPIDS OF *CHLAMYDOMONAS REINHARDI* UNDER DIFFERENT GROWTH CONDITIONS

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Abstract—Photo-, mixo- and heterotrophically grown cultures of *Chlamydomonas reinhardi* (wild type ss and 2 streptomycin-resistant mutants sr_3 and sr_{35}) have been analyzed for lipids and fatty acids. Ether-soluble lipids, chlorophyll, monogalactosyl diglyceride, digalactosyl diglyceride, sulfolipid, phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl glycerol and the relative amounts of fatty acids in total and individual lipids have been determined. The lipid and fatty acid compositions are very similar in the 3 strains and are not affected by the mutations. Fatty acids belong exclusively to the C_{16} and C_{18} series, 16:0, 16:4, 18:1, 18:2, 18:3 (6,9,12) and 18:3 (9,12,15) comprising about 90% of the total. 18:3 (6,9,12) is concentrated in phosphatidyl ethanolamine. In streptomycin-bleached sr_3 cells, ether-soluble lipids increase from 7 to 11% of dry weight on greening, mostly due to synthesis of monogalactosyl diglyceride and chlorophyll. Monogalactosyl diglyceride of bleached cells exhibits the same fatty acid pattern before and after greening.

INTRODUCTION

The unicellular alga *Chlamydomonas reinhardi*, is an organism suitable for investigating the formation of cell organelles [1] and for studies on the biogenesis of the chloroplast lamellar system [2,3]. For this purpose mutants are used, which differ from the wild type (ss) by resistance to streptomycin. Mutant sr_3 exhibits a chromosomal and mutant sr_{35} an extrachromosomal inheritance of resistance [4]. Strain sr_3 is an appropriate model for thylakoid formation, since it forms bleached cells in the dark in the presence of streptomycin, which develop thylakoids on illumination [5]. This approach requires first of all information about the lipid and protein constituents, which are integral parts of chloroplast lamellae. Analyses of fatty acid composition of different strains of *Chlamydomonas* [6] suggest considerable variation among different strains and species.

In this paper, lipid and fatty acid composition of the strains ss, sr_3 and sr_{35} have been analyzed under different growth conditions. Analyses have also been carried out with bleached cells of sr_3 on greening. Results reported here were obtained from whole cells, since, on the one hand, total lipids are also representative for chromatophores, and on the other hand, the isolation of pure chloroplast preparations from *Chlamydomonas* is still unsatisfactory.

Abbreviations: MGDG—monogalactosyl diglyceride; DGDG—digalactosyl diglyceride; SL—sulfolipid; PE—phosphatidyl ethanolamine; PG—phosphatidyl glycerol; PC—phosphatidyl choline; 18:3 (6,9,12)-γ-linolenic acid; 18:3 (9,12,15)-α-linolenic acid.

RESULTS

Lipids of green cultures

The content of ether-soluble lipids was investigated under phototrophic (nutrient without acetate, light), mixotrophic (nutrient with acetate, light) and heterotrophic (nutrient with acetate, dark) growth conditions. Under heterotrophic conditions, the lipid content is 19·5–24% of dry wt, as shown in Table 1. On addition of acetate, the percentage decreases slightly whereas streptomycin depresses the lipid content to 15% in sr_3, while sr_{35} is not affected. Heterotrophically grown cultures always possess a lower lipid content than photo- or mixotrophic cells. For ss this decrease comprises about 60% of dry wt, but only 40%, if based on the cell number. Part of the decrease must therefore be due to increased starch formation in bleached cells.

For the quantitative determination of individual lipids, the ether-soluble lipid was fractionated by TLC. Chlorophyll, MGDG, DGDG, SL, PE, PG, PC were determined as representative components. In the lipid extracts of all the strains, an acyl lipid comprising about 7–10% of the total was also present, which we could not identify and is therefore called lipid X. It occurs in fresh, as well as in lyophilized cells and seems therefore not to be an artifact. The structure of lipid X will be the subject of a following paper. The lipid pattern of the 3 strains is qualitatively identical for mixo- and heterotrophic conditions, as shown in Table 2. Minor differences between strains, however, are observed in the percentage of individual lipids. For heterotrophic cultures, an elevated MGDG/DGDG ratio appears to be typical. From the 24–30% of the total lipids, which were not characterized,

Table 1. Lipid content of *Chlamydomonas reinhardi* ss, sr_3 and sr_{35} under different growth conditions

	ether-soluble lipid (mg)					
	ss		sr_3		sr_{35}	
	per g dry wt	per 10^9 cells	per g of dry wt	per 10^9 cells	per g of dry wt	per 10^9 cells
Phototrophic	195	18·3	240	—	214	—
Mixotrophic	197	—	180	28·2	186	16·4
Mixotrophic + streptomycin						
5 μg/ml	—	—	138	—	217	—
10 μg/ml	—	—	163	—	206	—
20 μg/ml	—	—	162	—	204	—
40 μg/ml	—	—	140	—	190	—
Heterotrophic	87	11·0	78	17·0	117	10·5

Table 2. Chlorophyll and lipid content of *Chlamydomonas reinhardi* strains ss, sr_3 and sr_{35} under mixotrophic and heterotrophic conditions. Mixo/str. = mixotrophic growth in the presence of 20 μg/ml streptomycin sulfate

	% total ether-soluble lipid							
	ss		sr_3			sr_{35}		
	mixo	hetero	mixo	mico/str	hetero	mixo	mixo/str	hetero
Chlorophyll	18·8	10·3	21·1	—	13·7	24·3	—	17·6
MGDG	26·3	29·9	28·3	28·8	36·0	27·6	26·1	32·1
DGDG	19·3	14·9	13·3	14·3	9·4	12·6	20·9	12·9
PG	3·5	4·6	6·1	7·2	7·7	3·9	6·1	6·4
PE	2·5	44·6	4·4	6·8	3·4	3·4	3·4	4·0
PC	1·5	3·4	1·0	2·4	1·7	1·4	1·2	1·6
SL	2·0	2·3	2·2	1·0	2·5	1·4	2·3	1·6
U				24–30				

U = unidentified and uncharacterized components (including carotenoids, sterols, lipid X, etc.).

Table 3. Fatty acid composition of total lipid of *Chlamydomonas* strains ss, sr_3 and sr_{35} under different growth conditions

	wt % of total fatty acids							
	ss		sr_3			sr_{35}		
	mixo	hetero	mixo	mixo/str	hetero	mixo	mixo/str	hetero
14:0	tr	tr	tr	tr	tr	tr	—	tr
16:0	19·2	26·9	19·7	20·6	22·9	16·0	16·2	21·0
16:1(7) + (9)	2·2	2·4	2·9	3·5	4·6	2·5	3·4	2·6
16:1(3*t*)	1·2	0·8	1·3		1·0	1·5		0·9
16:2(7,10)	2·4	2·6	1·0	—	0·7	1·2	—	0·8
16:3	1·4	0·9	1·1	4·5	1·6	1·0	8·8	2·4
16:3	5·3	5·0	2·0	—	1·4	2·7	—	1·2
16:4	21·1	11·7	20·7	18·9	18·8	22·0	18·6	18·3
18:0	tr	tr	tr	2·3	tr	—	—	—
18:1	5·4	8·8	8·4	10·1	11·1	10·1	12·2	10·3
18:2	10·5	12·5	8·6	6·5	7·4	6·5	12·3	7·9
18.3(6,9,12)	8·7	9·1	10·0	9·6	11·4	8·1	9·2	9·5
18:3(9,12,15)	22·4	18·5	24·7	23·6	18·7	28·4	19·3	24·8
18:4	tr	tr	tr	tr	tr	tr	tr	tr
% Saturated fatty acids	19·2	26·9	19·7	22·9	22·9	16·0	16·2	21·0
% C_{16} Fatty acids	52·8	50·3	48·7	47·5	51·0	46·9	47·0	47·2

tr = trace.

one third consists of lipid X and the rest of carotenoids, sterols and other non-polar lipids. From Table 2 it is also evident, that streptomycin up to 20 μg per ml of nutrient does not influence the lipid pattern.

The fatty acid pattern is qualitatively identical and quantitatively very similar in all strains and for all growth conditions examined, as presented in Table 3. The dominant components belong to the C_{16} and C_{18} series, which occur in a weight ratio of about 1:1. The percentage of saturated acid is 16–27%, palmitic acid (16:0) is the main component. 16:0, 16:4, 18:1, 18:2, 18:3 (9,12,15) and 18:3 (6,9,12) acids constitute up to 90% of total fatty acids. The fatty acids listed in Table 3 were identified by GLC and MS, and in single cases, also by IR and by identification of silylated dihydroxy derivatives, obtained by oxidation.

The distribution of fatty acids among individual lipids of mixotrophic cultures is demonstrated in Fig. 1. Beside single variations between strains, some general tendencies are apparent. MGDG is characterized by large amounts of 16:4 and 18:3 (9,12,15) as well as by 16:3, 16:2 and 18:2 acids. In contrast, DGDG contains larger proportions of 16:0 and 18:1, and in the case of strain ss, also of 16:1, acids than the MGDG. 18:3 (6,9,12) acid is absent from glycolipids, but in phospholipids, however, large amounts of the acid are present, while neither 16:4, nor 18:3 (9,12,15) acids occur. In strain ss, 18:3 (6,9,12) comprises 37% of the total fatty acids and appears to be generally located in PE, which is also the only lipid containing considerable amounts of 18:0 acid. PG is characterized by a large amount of 16:1, and PC by large amounts of 18:1 acids.

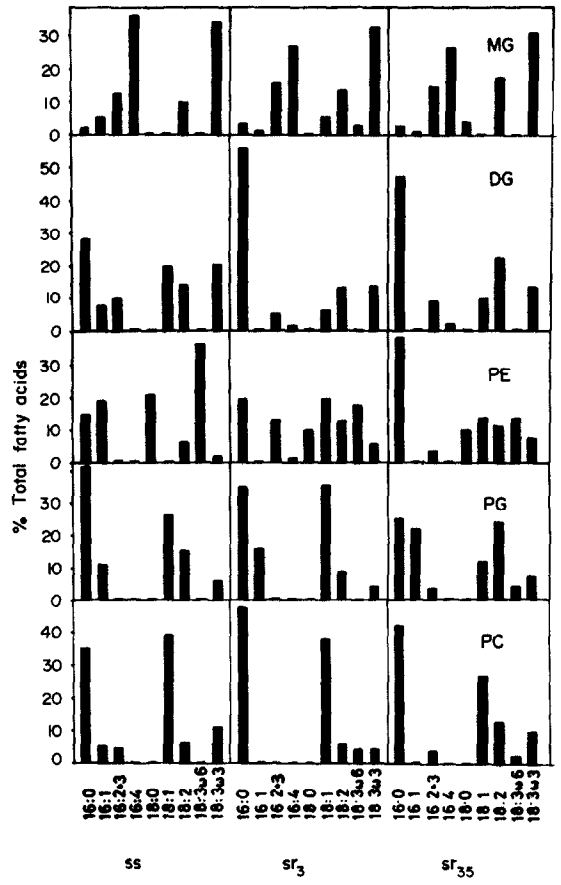

Fig. 1. Fatty acid distribution among individual lipids of mixotrophic cultures of *Chlamydomonas reinhardi* ss, sr_3 and sr_{35}.

Table 4. Chlorophyll and lipid content of streptomycin-bleached *Chlamydomonas* sr_3 cultures before and after greening (48 hr)

	wt % Ether-soluble lipids	
	Bleached	After 48 hr of greening
Chl	3·4	14·5
MGDG	18·1	20·9
DGDG	13·9	7·8
PG	6·1	4·0
PE	5·3	3·9
PC	3·0	1·9
SL	2·3	2·0
Ether-soluble lipids (% dry wt)	7·3	11·1

Lipids of streptomycin-bleached and greening cells

The lipid composition, as shown in Table 4, shows that bleached cells contain about 7%, those illuminated for 48 hr [5] 11% ether-soluble lipids based on dry wt. Although the increase of chlorophyll depresses the relative amounts of the other lipids, formation of MGDG is obvious on illumination, raising the MGDG/DGDG ratio to a level similar to that found in mixotrophic cultures (Table 2). The total of the identified compounds only accounts for *ca* 52–55% of the total lipids in bleached cells compared to 70–76% in green cells. The reason for this difference is not yet clear.

The fatty acid composition of the total and individual lipids, as shown in Fig. 2, demonstrates, that during greening the relative amounts of 16:0, 18:2 and 18:3 (6,9,12) acids decrease, while the 16:4, 18:1 and 18:3

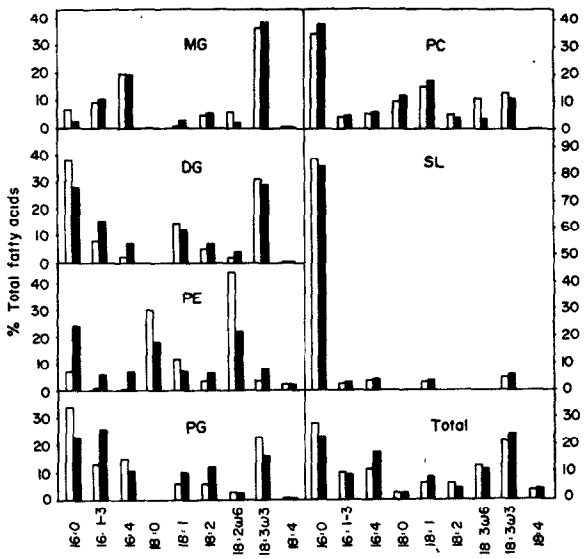

Fig. 2. Fatty acid distribution in total and individual lipids of streptomycin-bleached *Chlamydomonas reinhardi* sr_3 cells before (□) and after (■) greening.

(9,12,15) acids correspondingly increase. This augmentation of trienoic and tetraenoic acids, which are mainly localized in MGDG, is in accordance with the above mentioned synthesis of this lipid. The composition of individual lipids is affected by the change of the total fatty acid pattern in different ways. In MGDG, as well as in SL and PC, no marked change occurs. In contrast, DGDG is characterized by a significant increase in the amount of C_{16} polyenoic acids, while in PG 16:1 (mainly 3-*trans*) acid increases. On the other hand, PE of greening cells contains much more saturated and unsaturated acids of the C_{16} series than PE of bleached cells, where 18:0 and 18:3 (6,9,12) acids predominate in this lipid.

DISCUSSION

The present results demonstrate that, as regards the important lipid components of photosynthetic membranes, the 3 strains of *Chlamydomonas* examined are identical. This means that the genetic alterations leading from the wild type ss to the mutants sr_3 and sr_{35} have no effect on the nature of membrane lipids, nor has the addition of streptomycin to the medium.

Compared with other strains of species of *Chlamydomonas*, some differences in the fatty acid pattern are evident. Our strains form 16:4 acid in considerable amounts, as does the one analyzed by Bloch [7]. In contrast, a 16:3 acid appears to be typical for *C. mundana* [8]. In other strains [9], 18:3 and 20:1 acids predominate instead of 16:4. Since polyenoic acids of the C_{16} and C_{18} series are predominantly localized in MGDG and therefore in the chloroplasts [10], as shown also by our experiments, it follows that the fatty acids of chloroplast lipids may vary considerably among different species and strains. Whether the differences are due to differing capabilities in fatty acid synthesis or simply to the activity of specific acyl transferases, has not been elucidated in detail. Interestingly, a proportion of 18:3 (6,9,12), even larger than in other strains of *Chlamydomonas* [9,11] appears to be typical of our organism. This fatty acid is more characteristic for heterotrophically grown, than for autotrophically grown organisms, and is concentrated in non-photosynthetic membranes of the cell [6]. This is in accordance with the fact that the relative amount of 18:3 (6,9,12) acid is enhanced in dark-grown cultures. Strikingly, in our experiments it is found to be concentrated in PE. Similar results were obtained also with *Ochromonas danica* [10]. The preference of PE for 18:3 (6,9,12) could signify that this lipid is involved in the synthesis of this acid. Similar functions are known for PC in further desaturation of 18:1 acid [12] and for PG in formation of 16:1 (3*t*) from 16:0 acid [13]. Despite the fact that PE seems to be a minor lipid constituent of chloroplasts [14], mostly localized outside the chromatophores, the possible role of this lipid in the synthesis of 18:3 (6,9,12) acid has to be further investigated. Although in general not typical for chromatophores, 18:3 (6,9,12) occurs in considerable amounts in chloroplasts of *Ochromonas danica* [10] and is probably formed from linolenic (18:2) acid, as demonstrated in blue-green algae [15]. Further experiments are hindered by the fact that isolation of chloroplast preparations from *Chlamydomonas* is still unsatisfactory, due to contamination by other cell organelles and to partial lipid breakdown by degrading enzymes.

There is also the question to be resolved about where such typical chloroplast lipids as MGDG or carotenoids are localized in bleached cells, which do not contain thylakoid structures. Interestingly, MGDG of bleached cells have about the same fatty acid pattern as MGDG of green cultures, demonstrating that bleached cells are able to provide the complete set of acyl groups necessary to synthesize the type of MGDG needed for functional chloroplasts.

EXPERIMENTAL

Cultivation of organisms. *Chlamydomonas reinhardi* (wild type ss and the mutants sr_3 and sr_{35}) [16] were cultivated as described [4,5]. The nutrient was medium I [26] with 0·1 g arginine hydrochloride added per l. In mixotrophic and heterotrophic cultures the medium contained in addition 2 g NaOAc . 2 H_2O per l. Photo- and mixotrophic cultures were grown under 10 000 lx of fluorescent light for 3 days at 26–28°, heterotrophic cultures for 10 days at the same temp in the dark. Streptomycin-bleached cultures of sr_3 were cultivated as described in ref. [5].

Extraction of lipids. 1–2 g of cells were mixed with 10 vols of $CHCl_3$–MeOH (1:1) and heated to 70° for 30 sec. Aliquots of the suspension were used for the determination of dry wt and chlorophyll [17]. From the residue lipids were extracted with Et_2O.

Preparation of fatty acids. Et_2O-soluble lipids were refluxed with 50 vols of MeOH–HCl 3% (w/w) for 1 hr and the crude ester fraction extracted with petrol. Crude esters were hydrolyzed with 20 vols of KOH–H_2O–EtOH (1:2:20) (w/v/v) for 1 hr. After removing insaponifiable material fatty acids were recovered with petrol and re-esterified with MeOH–HCl. Preparative separation of C_{16} and C_{18} acids was carried out by GLC on a FID instrument equipped with a stream splitter. A stainless steel column 1·8 mm × 4 mm i.d., packed with 15% DC 200 isothermal 210°, N_2 at 120 ml/min, was used. Both fractions were further fractionated according to unsaturation by TLC on Si gel G–$AgNO_3$ (19:1) with petrol–Et_2O (3:1). Fractions eluted with Et_2O were finally purified by preparative GLC on a 1·8 m × 4 mm i.d. column packed with 15% DEGS, isothermal 175°, N_2 at 30 ml/min.

Identification of fatty acids. 14:0, 16:0, 18:0, 18:1, 18:2 and 18:3 (9,12,15) acids were identified by GLC as Me esters by RR_f comparison with authentic reference compounds on 1·8 m × 2 mm i.d. column packed with 5% DEGS, isothermal 144°, N_2 at 15 ml/min. Chain length of unsaturated compounds was determined by GLC after hydrogenation. Evidence for the number of double bonds was obtained from R_f values of compounds on $AgNO_3$–Si gel TLC plates relative to known mono-, di- and trienes, and also by their GLC RR_f values relative to saturated chains. The degree of unsaturation was confirmed by MW's determined by MS. C_{16} monoenes yielded $M^+ = 268$, the diene $M^+ = 266$, trienes $M^+ = 264$, and the tetraene $M^+ = 262$. Positions of the double bonds in C_{16} monoenes were confirmed by oxidation of the fatty acid Me ester to the corresponding diol and by MS of the corresponding TMSi ether derivative [18,19]. Me esters were also cleared by oxidation [20] and the resultant monocarboxylic acid identified by GLC. Both methods confirmed the existence of 16:1(9), 16:1(7) and 16:1(3*t*) acids. The *trans* configuration of the latter was established by IR absorption at 1000 cm^{-1}. The C_{16} diene was identified as 16:2(7,10) acid [18]. 18:3(6,9,12) Me ester yielded $M^+ = 292$, and 18:4 ester $M^+ = 290$. GLC RR_f values of both compounds are in accordance with values obtained under similar conditions [9].

TLC of lipids. Aliquots of Et_2O-soluble lipids were separated on Si gel G and vizualized by 2',7'-dichlorofluorescein. The following solvents were used: (A) $CHCl_3$–MeOH–7N NH_4OH (65:30:4) [21], (B) $CHCl_3$–MeOH–HOAc–H_2O (170:25:25:6) [21] and (C) $CHCl_3$–MeOH–EtOAc–2% NH_3

(50:25:25:1·5) [22]. MGDG, DGDG and SL were separated by solvent C and determined by the anthrone procedure [23]. PC, PE and PG were separated by solvent B, charred with $HClO_4$ and determined as Pi [24]. Pure lipids in mg quantities were isolated by subsequent TLC with solvents A and C (MGDG, DGDG, SL) and solvents A and B (PC, PE, PG). Fatty acid Me esters were prepared with Na-methoxide [25].

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