COMPOSITION OF THE NEUTRAL LIPIDS OF Chlorella vulgaris

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The composition of chlorella lipids has been discussed in many publications. The fatty-acid composition of the total lipids [1, 2] and of the pigment complex of the chlorella lipids [3, 4] has been determined, and the influence of the change in the conditions of growing the alga on the composition of the fatty acids in the total lipids has been studied [5-8]. In the total lipids of Chlorella pyrenoidosa, the classes of neutral lipids that have been detected comprise triglycerides, free fatty acids, sterols, and pigments [9]. However, there are considerable differences in the methods of isolating the total lipids, in the choice of extractants, and in the selection of the initial sample (aqueous suspension of algae, freeze-dried or freshly isolated cells). Ivanova and Popov [10] have studied the influence of the conditions of isolation (solvents, form of the sample, extractants) on the quantitative yield of the total lipid. By combining various factors, they developed the optimum conditions for the quantitative isolation of the lipids from the microalgae.

In the present paper we present the results of a comparative study of methods of isolating the total lipids as a function of the nature of the sample, and also the composition of the total neutral lipids (NL's) and the fatty-acid composition of the individual classes of the NL's of Chl. vulgaris. Samples of fresh, freeze-dried, and air-dried algal cells were investigated.

The lipids were extracted with chloroform–methanol (2:1) from fresh cells (FC's) of chlorella after their centrifuging and from lyophilized cells (LC's). Extraction of the air-dried cells (ADC's) was carried out first with diethyl ether (ADC-1) and then with chloroform–methanol (ADC-2). The amount of lipids isolated from the extracts, calculated on the weight of the dry matter was 14% for the FC's, 12.6% for the LC's, 7.6% for the ADC-1, and 6.6% for the ADC-2. Diethyl ether extracted from the air-dry cells approximately half the lipids present in them, which is due to the capacity of this solvent for extracting only weakly bound lipids; a mixture of chloroform and methanol extracted the strongly bound lipids.

The lipid extracts were analyzed in relation to classes by thin-layer chromatography (TLC) on Silufol plates in system 1. The classes were identified on the basis of literature information [11], and also by comparison with model samples: cottonseed oil, fatty acids, β-sitosterol, and the methyl esters of the fatty acids of cottonseed oil. Hydrocarbons (HC's, R_f 0.98), sterol esters together with carotenoids (SE's + C's, R_f 0.90), fatty acid methyl esters (FAME's, R_f 0.76), triglycerides (TG's, R_f 0.60), free fatty acids (FFA's, R_f 0.39), free sterols (FS's, R_f 0.19), and four unidentified fractions with R_f 0.30, 0.14, 0.12, and 0.05 were found in all the extracts.

To isolate the classes of NL's, the lipid extracts from the FC's and the ADC-1 were chromatographed on a column of silica gel using a known sequence of solvents [11]. The ratio of the fractions was determined gravimetrically. The separation was monitored by TLC under the conditions described. Separation of the FC extract gave the following fractions (%): HC's + pigments + SE's – 17.5; SE's + Cs – 2.0; FAME's – 1.2; TG's – 16.4; FFA's – 7.1; FS's – 12.0; unidentified polar fractions – 43.8. The polar fractions were eluted together with the pigments, which, presumably, on the basis of their green color, can be assigned to the chlorophyll group [4].

When the extract from the ADC-1 was chromatographed on a column, it was possible to isolate only fractions enriched with various classes of NL's, the fractions from the AC's to the FFA's being eluted from the column with pure hexane. Analysis of this eluate by TLC showed that in the sum of the ADS-1 NL's the FFA's predominated. It is obvious that when the sample of chlorella from which the ADC-1 extract was obtained was dried, the triglycerides underwent cleavage to FFA's as the result of enzymatic reactions.

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TABLE 1. Compositions of the Fatty Acids of the Neutral Lipids of Chlorella vulgaris (%, GLC)

Acid	Extract of fresh cells				Total of the extracts		
	total	FAME's	TG's	FFA's	FC's	ADC-1	ADC-2
X_1	0,1	—	—	—	0,2	0,1	0,1
10:0	0,1	—	—	—	0,1	0,1	0,1
10:x	—	—	—	—	—	—	0,2
12:0	0,1	—	—	—	0,1	0,1	0,3
14:0	0,4	—	0,1	0,2	0,3	0,2	0,4
15:0-iso	—	—	—	—	—	—	0,5
14:1	0,3	—	—	—	0 2	—	0,7
14:2 (15:0)	0,3	—	—	—	0,2	0,2	—
16:0	12,9	59,6	5,0	5,8	12,6	11,1	18,4
16:1 (17:0-iso)	6,3	16,0	3,2	6,8	6,2	5,3	6,7
16:2	1,7	1,7	1,4	1,3	1,6	1,4	2,0
X_2	5,3	—	10,2	5,1	5,3	4,5	4,9
16:3 (18:0)	18,5	—	24,2	19,5	19,8	18,2	15,0
18:1	8,2	16,1	3,0	3,2	8,0	8,6	8,9
18:2	7,4	6,6	8,5	6,5	8,0	9,1	8,2
18:3	34,9	—	42,2	42,3	33,8	36,4	31,6
20:0	3,6	—	2,2	5,3	3,6	4,7	2,0
20:1	—	—	—	4,0	—	—	—

The FAME fraction of the lipid extract of the FC's was reseparated on a column of silica gel to free it from accompanying components. The degree of purity was checked by TLC. The FAME's isolated were investigated further by chromatography (GLC). To confirm that this class of compounds actually consisted of FAME's, they were converted into the FA's by saponification, the formation of the fatty acids being checked by TLC. After methylation with diazomethane, the re-formed FAME's were re-investigated by GLC. The identity of their composition before and after saponification shows that the FAME's are present in the total natural lipids.

The TG fraction of the FC extract was saponified, the fatty acids were isolated, and, after methylation, they were analyzed by GLC. The FFA fraction isolated from the same extract was analyzed by GLC after methylation.

The combined fatty acids from the extracts of the total lipids of the FC's, LC's, ADC-1, and ADC-2 were isolated similarly, with the separation of the unsaponifiables. The fatty-acid compositions of all the fractions are given in Table 1; the acids are given in the order of their elution.

In GLC, the acids were identified from literature characteristics [1, 2], with the aid of an internal standard ($C_{18:0}$), on the basis of a comparison with markers, from a graph of the dependence of the logarithm of the retention volume on the number of carbon atoms in the chain of the acid, and from the relative retention volumes [12, 13].

The quantitative compositions of the fatty acids of the lipid extracts of the samples that we investigated practically coincided. In the quantitative respect, the unsaturated acids of the C_{16} and C_{18} series predominated. In their quantitative composition, the fatty acids of the FC, LC, and ADC-1 extracts were similar, while the ADC-2 extract was characterized by an increased amount of palmitic acid, $C_{16:0}$, and a lower amount of linolenic acid, $C_{18:3}$. In contrast to the TG and FFA fractions of the FC extract, consisting mainly of unsaturated acids, the FAME fraction contained 59.6% of palmitic acid.

The chromatograms of the ME's of the acids of all the extracts showed combined peaks containing more than one acid. To refine the identification and to determine the position of the first double bond in the main unsaturated acids we performed the destructive oxidation of the total FFA's of the ADC-1 extract. The following acids were identified in the degradation fragments: initial unsaturated $C_{18:0}$ – 2.0; $C_{17:0}$-iso – 1.0; $C_{16:0}$ – 34.9; $C_{15:0}$ – 0.2; $C_{14:0}$ – 0.2; $C_{12:0}$ – 1.0; newly formed $C_{9:0}$ – 3.5; $C_{13:0}$ – 1.0; adipic (C_6) – 3.4; pimelic (C_7) – 2.0; suberic (C_8) – 2.6; azelaic (C_9) – 43.9; and unidentified X_3 and X_4 – 2.3 and 2.0, respectively. These results confirmed that the peak corresponding to the $C_{18:0}$ acid of the chromatogram was a combined peak consisting of the $C_{18:0}$ and $C_{16:3}$ acids with the latter predominating. The main unsaturated acids had their first double bond in the Δ^9 position, but it may also be assumed that in a number of acids the center of unsaturation was located at Δ^3, Δ^6, Δ^7, or Δ^8 [14, 15], and that the unidentified acid X_2 was unsaturated. It is known that the total acids of chlorella include the Δ^3–16:1 acid [14].

The investigations performed have shown that, although the quantitative yields of the lipid extracts from the FC, LC, and ADC samples differ slightly, the lipids are isolated better from the fresh cells in which the material is present in the native state.

To identify the pigments in the ML's of the FC extract, the UV spectra of the colored lipid fractions eluted from the column were recorded. The pigments issuing from the column together with the hydrocarbons had adsorption in the UV spectrum at λ_{max}^{hexane} 464, 655 nm, which permits them to be assigned to chlorophyll b. With the FAME's were eluted pigments with absorption in the UV spectrum at λ_{max}^{hexane} 270, 340, 446, 424, and 474 nm (β-carotene); with the FFA's – at λ_{max}^{hexane} 226, 406, 428, 452, 478 nm (4-oxo-α-carotene); and with the free sterols – at λ_{max}^{hexane} 412, 452, 508, 536, 616, 650, and 676 nm – the pigment chlorophyll *a* [4, 16, 17].

EXPERIMENTAL

The UV spectra were taken on a Hitachi instrument in the 200-700 nm region in hexane. The alga Chlorella vulgaris (local strain) was grown in pan-type apparatuses under the open sky in 0,4 medium [sic] and was aerated with air containing 1-5% of CO_2 [18], the cell density amounting to 19 million/ml. The temperature during the cultivation period (12 days) varied from 18 to 30°C. After the yield of the culture had entered onto a plateau, the suspension was separated.

The lipids were isolated from the fresh cells directly after centrifuging an aqueous suspension of the biomass (6000 rpm); from the freeze-dried (lypohilized) biomass after 8 days; and from the air-dry mass 18 days after the isolation of the fresh cells. In all cases, the lipids were extracted by steeping with the extractant at a low temperature five times. The crude extracts were washed with a chloroform–methanol–water (8:4:3) solvent mixture [12] and were evaporated in vacuum.

The lipid extracts (5 g each) of the FC's and the ADC-1 were fractionated on a column (65 × 2 cm) containing type KSK silica gel that had been treated by the method of Akhrem and Kuznetsova [19] (48 g, particle size 0.16 mm). From the lipid extract of the FC's, hexane (150 ml) eluted hydrocarbons, sterol esters, and pigments; hexane–diethyl ether (99:1; 100 ml) eluted the fatty-acid methyl esters; hexane–diethyl ether (90:5; 200 ml) eluted the triglycerides; hexane–diethyl ether (90:10; 150 ml) eluted the free fatty acids; diethyl ether (70 ml) eluted the free sterols; chloroform (50 ml) eluted two unidentified fractions; and methanol (200 ml) also eluted two unidentified fractions.

Qualitative TLC was performed on Silufol plates in the heptane–methyl ethyl ketone–acetic acid (41:9:0.5) system (1) [20], and the rechromatography of the FAME's on a column (50 × 1 cm) of silica gel (15 g) with elution by a mixture of hexane and diethyl ether (98:2).

The fatty acids were isolated from the lipid extracts from the individual classes of NL's (TG's, FAME's) by saponifying the samples with 10% methanolic KOH solution at room temperature. In the isolation of the FA's from the lipid extracts, the unsaponifiable substances were separated by extraction with diethyl ether [21]. The combined fatty acids were freed from pigments by chromatography on silica gel, the FA's being eluted with hexane–diethyl ether (90:10; 200 ml). The acids were methylated with diazomethane. The FA methyl esters were analyzed on a UKh-2 chromatography under the conditions described previously [22].

Oxidation with Periodate-Permanganate [23]. A mixture of 0.1 g of FFA's, 40 ml of tert-butanol, 60 ml of water, 1.7000 g of KIO_4, 0.0160 g of $KMnO_4$, and 0.0550 g of K_2CO_3 was boiled on the water bath under reflux for 1 h. After cooling, 10% H_2SO_4 was added to an acid reaction, the excess of oxidizing agent was decomposed with dry $Na_2S_2O_5$, the acidic fragments were converted into soaps by the addition of KOH, the tert-butanol was distilled off, the soaps were decomposed with 15% HCl, and after saturation with NaCl the acid were isolated by extraction with diethyl ether. After elimination of the solvent, the acids were methylated with diazomethane and were analyzed by GLC.

SUMMARY

The qualitative and quantitative compositions of the neutral lipids and the fatty-acid composition of the individual classes of neutral lipids isolated from fresh, freeze-dried, and air-dry cells of the alga Chlorella vulgaris have been investigated.

The presence of natural fatty acid methyl esters in the lipid extract of the fresh and air-dry chlorella cells has been established.

It has been shown that the fatty acid methyl ester fraction is enriched with palmitic acid as compared with the triglyceride and free fatty acid fractions.

The main unsaturated acids of the chlorella lipids contain the first double bond in the Δ^9 position.

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STUDY OF THE PHOSPHOLIPIDS OF VARIOUS ORGANS OF *Crambe amabilis* ACCORDING TO THE VEGETATION PERIODS

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The known studies of phospholipids according to the vegetation periods of plants are devoted mainly to the changes in the phospholipid complex in the process of the ripening of the seeds of certain plants [1-6]. Our aim was to study the change in the fatty-acid and fractional compositions of the phospholipids of various organs of *Crambe amabilis* according to the vegetation phases of the plant.

The plants were collected in 1977 in the Bostanlyskii region of the Tashkent oblast (environs of Burchmulla). The combined phospholipids (PL's) from the various organs of the plant [7, 8] were freed from accompanying carbohydrates [9] and were analyzed in a thin layer of silica gel in solvent systems 1 and 2. From their chromatographic mobilities, the phospholipids with R_f 0.4 and 0.98 (in system 1) and 0.3 and 0.9 (in system 2) were assigned, respectively, to the phosphatidylglycerols (PG's) and the phosphatidic acids (PA's), which are widely distributed in the leaves of plants [1, 2]. The amounts of the individual PL's (Table 1) were determined from the phosphorus contents [10] of the corresponding spots on the chromatogram [11].

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