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Regulation of lipid biosynthesis in *Chlorella kessleri* by clomiphene

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During autotrophic cultivation of the alga *Chlorella kessleri* clomiphene[1]) regulated both the quantitative and qualitative production of lipids (sterols, hydrocarbons and fatty acids). Their content decreased to 57% as compared with the production of sterols in cultures without clomiphene. The production of hydrocarbons and fatty acids was similar to that on the control.

Among the sterols the variety of which enhanced the presence of clomiphene, those prevailed which carried either a double bond in position 8 or were devoid of it in position 22. In the mixture of hydrocarbons a significant increase was observed towards n-heptadecane production. Oleic acid predominated among fatty acids.

After initial experiments in vertebrates (Blohm *et al.* 1959, Avigan *et al.* 1960) the study of the inhibition of lipid biosynthesis by taking sterols as a model extended to plants and microorganisms (Douglas and Paleg 1981) including algae of the genus *Chlorella* (Patterson *et al.* 1973). These studies clarified both the sequence of sterol biosynthesis (Chan *et al.* 1974) and the mechanism of action of inhibitors, e.g. fungicides (Gadher *et al.* 1983).

In the present work we studied in addition to proteins and polysaccharides the regulation of the biosynthesis of lipids which belong to the most important compounds of green algae. The alga *C. kessleri* served as a model microorganism. Clomiphene was used as inhibitor.

Materials and methods

Cultivation: *C. kessleri* was autotrophically cultivated for 9 days at 33 °C in a tubular cultivator (volume 66 l, total illuminated area of 32 mm tubes — 5 m^2). During cultivation the light intensity was gradually regulated up to a maximum of 220 W/m^2 of photosynthetically active radiation. The algal suspension was recirculated with a centrifugal pump and saturated with an air — carbon dioxide mixture. Control cultivation was without the inhibitor. The concentration of clomiphene was 3.08 mg/l. After completion of the cultivation the algae were separated by centrifugation.

Isolation and separation of lipid fraction: 10 g of the algal suspension were boiled for 15 min with 0.5 M aqueous NaOH. The unsaponifiable fraction was extracted three times with 50 ml of diethyl ether, washed with water, dried and evaporated to dryness. It was then separated by preparative TLC (silica gel H) in hexane — diethyl ether into sterol and hydrocarbon fractions. The residue after diethyl ether extraction was acidified to pH 2 with HCl, fatty acids were extracted with 3×50 ml of diethyl ether and converted to methyl esters (BF_3-methanol) and pyrrolidides (Andersson 1978).

Gas chromatography — mass spectrometry: Mixtures of hydrocarbons and sterols in the form trimethylsilyl ethers and of fatty acids as methyl esters and pyrrolidides were analyzed on fused silica capillary columns in a Hewlett-Packard 5995 B (CA, USA).

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[1]) Clomiphene is the active component of the antiestrogenic preparation Gravosan (Spofa, Czechoslovakia); 2-/4-(2)chloro-1,2-diphenylethenyl)-phenoxy/-N,N-diethylethanamine

Results

Table 1 summarizes data on the production of individual lipid fractions in the alga cultivated with and without clomiphene.

Fatty acids were identified and quantified by gas chromatography and mass spectrometry of methyl esters (Řezanka *et al.* 1983). The positions of double bonds were determined in pyrrolidides according to Andersson (1978). After the application of clomiphene the content of oleic acid (18:1) increased significantly, while a slight shift towards lower values occurred for 16:0 and 16:1 acids (Table 2). The content of 18:2 and 18:3 acids decreased substantially whereas 16:2 and 16:3 acids and those above C_{20} disappeared.

In mass spectra hydrocarbons were identified on the basis of splitting. In a *C. kessleri* culture with clomiphene *n*-heptadecane was identified as the major hydrocarbon (97 relat. %). Sterols were identified on the basis of retention times (Patterson 1971) and interpretation of mass spectra (Brooks *et al.* 1973, Knights 1967). As shown in Table 3 15 sterols were detected in the alga cultivated with clomiphene. Three of them were not identified. On the other hand, in the culture without clomiphene 6 sterols were found and only one of them was not identified.

Table 1
Weight of fraction in *C. kessleri* (mg/10 g of suspension)

Lipid	Cultivation with clomiphene	Control cultivation
hydrocarbons	6	7
sterols	167	291
fatty acids	970	950

Table 2
Effect of clomiphene on the fatty acid composition in the alga *C. kessleri* (%)

Fatty acids	Cultivation	
	Control	With clomiphene
14:0	7.2	0.4
14:1ω^9	1.0	2.4
14:3ω^3	0.0	0.8
16:0	30.6	20.7
16:1ω^9	6.6	3.9
16:2ω^6	0.7	0.0
16:3ω^3	1.3	0.0
17:0[a]	6.4	8.1
18:0	8.8	3.3
18:1ω^9	7.0	41.8
18:2ω^6	19.6	1.7
18:3ω^3	7.6	0.2
20:0	0.4	0.0
minor and non-identified above	0.8	6.7
	3.0	0.0

[a] 2-methylhexadecanoic acid

Table 3
Effect of clomiphene on the sterol composition in the alga *C. kessleri* (%)

Sterols	Cultivation	
	Control	With clomiphene
5α-ergost-8-en-3β-ol	0.0	5.7
5α-ergost-8(14)-en-3β-ol	4.3	8.6
5α-ergost-7-en-3β-ol	50.3	20.6
5α-ergosta-8,22-dien-3β-ol	0.0	2.8
5α-ergosta-8(14),22-dien-3β-ol	0.0	3.4
5α-ergosta-7,22-dien-3β-ol	2.8	0.0
5α-ergosta-5,22-dien-3β-ol	0.0	4.1
14α-methyl-5α-ergosta-8,22-dien-3β-ol	0.0	5.6
5α-stigmast-8-en-3β-ol	0.0	12.9
5α-stigmast-8(14)-en-3β-ol	9.3	2.9
5α-stigmast-7-en-3β-ol	23.8	15.4
5α-stigmast-5-en-3β-ol	0.0	3.3
5α-stigmasta-7,24(28)-dien-3β-ol	0.0	10.5
unknown A	6.8	0.0
unknown B[a]	0.0	2.1
unknown C[a]	0.0	1.4
unknown D[a]	0.0	0.4

[a] estimated by SIM method (single ion monitoring)

Discussion

Clomiphene regulated the growth of *C. kessleri*. Its optimal concentration required for a biomass increase comparable to that in the alga cultivated without clomiphene was 3.08 mg/l. Higher clomiphene concentrations inhibited the growth.

It follows from the results obtained that the addition of clomiphene to the alga *C. kessleri* resulted in two types of regulation of biosynthesis which affected either the amount of sterols produced or the composition of sterols, hydrocarbons and fatty acids.

Table 1 illustrates the changes in the production of individual components of the lipidic fraction. Whereas the production of hydrocarbons and fatty acids remained unchanges in cultures with or without clomiphene the total sterol production by algae cultivated in the presence of clomiphene decreased to 57% of that of the control. This finding indicated that clomiphene resembled in its mechanism of action citrinine — the classical inhibitor of sterol biosynthesis (ENDO and KUBODA 1976) (as compared with the other known inhibitor cerulenine-inhibiting biosynthesis of both fatty acids and sterols; VANCE *et al.* 1972). Since sterol production was decreased while the production of hydrocarbons and fatty acids remained unaffected it seemed likely that clomiphene inhibited a biosynthetic pathway proceeding from the polyketide pathway.

The second type of regulation was manifested by a qualitative and quantitative shift in occurrence of individual representatives of components of the lipid fraction studied. The increased production of fatty acid 18:1 and the decreased production of 18:2 and 18:3 acids was of primary importance. A similar relationship could also be observed between the production and the increasing reduction in the degree of saturation of C_{18} acids (Table 2). With respect to the biosynthesis of unsaturated fatty acids it may be assumed that clomiphene inhibited a dehydrogenation of fatty acids with single double bonds (THIELE 1979).

Frasinel *et al.* (1978) studied the effect of triarimol on fatty acid production in three species of the genus *Chorella* and described a similar composition of the mixture of fatty acids as in control cultivation, but the production of fatty acids increased up to ten times.

A rich mixture of hydrocarbons was found in algae cultivated without the inhibitor (Paoletti *et al.* 1976, Řezanka *et al.* 1982). The regulation of *n*-heptadecane production by clomiphene favours the assumption that its biosynthesis occurs by condensation of two lower acids to a C_{18} acid and is accompanied by a simultaneous decarboxylation and production of a hydrocarbon shortened by one carbon atom (McInnes *et al.* 1980). In the alga *Scenedesmus quadricauda* *n*-heptadecane (54.4%) was also found to be the major hydrocarbon when standard cultivation was applied (Stránský *et al.* 1968).

The main effects of clomiphene were: the inhibition of splitting of methyl in position 14, the shift of a double bond from position 8 to position 7, and the introduction of a double bond at position 22 of sterols. These data were in agreement with those obtained with the inhibiting compounds AY-9944 or triparanol (Patterson *et al.* 1973). Besides that, clomiphene increased the content of sterols with a double bond in position 5 and 24/28/ which may possibly be explained in case of position 5 by the stimulation of an isomerase. The addition of clomiphene resulted in a substancially increased content of those sterols which are precursors of the last steps of their biosynthetic pathway. On the other hand, an influence on double bond 8/14/ reduction and secondary alkylation on C_{24} was not observed.

Thus, in spite of similarities in the chemical structure of triparanol and clomiphene they influenced different steps of lipid biosynthesis.

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