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EFFECT OF TRIARIMOL ON STEROL AND FATTY ACID COMPOSITION OF THREE SPECIES OF *CHLORELLA**

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Key Word Index—*Chlorella sorokiniana, C. ellipsoidea, C. emersonii*; Chlorococcales; algal fatty acids; algal sterols; triarimol; sterol biosynthesis inhibitors.

Abstract—The concentration of triarimol giving *ca* 50% inhibition of growth was different for each of 3 species of *Chlorella* [*C. emersonii*, 1 mg/l. (1.5×10^{-6} M), *C. ellipsoidea* 10 mg/l. (3×10^{-5} M), *C. sorokiniana*, 2 mg/l. (6×10^{-6} M)]. The total lipid of 3 species of *Chlorella* grown in a culture medium containing triarimol were analysed for chlorophyll, fatty acids and sterol composition. Growth rates were studied in the presence of different concentrations of triarimol. The growth rates of the 3 species were differentially inhibited by triarimol. The growth of *Chlorella sorokiniana* was 50% inhibited by 2 mg/l. triarimol but 20 mg/l. did not produce a cessation of growth. The greatest inhibition of growth rates and chlorophyll content was observed in *Chlorella emersonii*. The quantity of unsaturated fatty acids was increased by triarimol treatment in all 3 species of *Chlorella*. Triarimol strongly inhibited 14α-demethylation in *Chlorella emersonii*, and *C. ellipsoidea* and less in *C. sorokiniana*, resulting in accumulation of 14α-methyl sterols. Triarimol also inhibited the second alkylation of the side chain in *C. ellipsoidea* and *C. emersonii*. The introduction of the 22-double bond was inhibited in all 3 species of *Chlorella* studied. Although some differences were apparent, the effect of triarimol was quite similar to that of triparanol and AY-9944 in these 3 species of *Chlorella*.

INTRODUCTION

A synthetic pyrimidine analogue α-(2,4-dichlorophenyl)-α-phenyl-5-pyrimidine methanol (Triarimol), is one of the main inhibitors studied in relation to the antifungal effects or interference with sterol metabolism in fungi [1–4]. It is structurally similar to triparanol, a drug which has been studied especially in relation to inhibition of sterol biosynthesis in several algae [5–9].

In rat liver subcellular fractions, triarimol inhibits 14α-demethylase, and has a slight inhibitory effect on Δ^7-sterol-Δ^5-dehydrogenase. A result of triarimol inhibition was also an accumulation of lanosterol and dihydrolanosterol during cholesterol biosynthesis from acetate and mevalonic acid [10].

Due to similarities in behavior of carbon monoxide and triarimol on 14α-demethylase, it appears that triarimol may be a specific inhibitor of cytochrome P-450 which participates in one or more reactions, leading to the oxidative elimination of the 14α-methyl group [10, 11]. The blocking of 14α-demethylase in rat liver [10] and *Ustilago maydis* [1] indicates a similarity between enzymes participating in 14α-demethylation of these two organisms. The effects of triarimol on ergosterol biosynthesis could account for its toxicity to *Ustilago maydis* [3].

Morphological effects due to triarimol treatment have been observed in sporidia of *Ustilago maydis* which fail to undergo more than one doubling or become branched and multicellular [3]. Triarimol-treated higher plants developed darker green leaves and raised areas between the veins [4]. Triarimol retarded shoot and root elongation, caused an increase in fresh weight, and abscission of leaves and flowers [12]. It appeared that triarimol and its analogue ancymidol, caused growth retardation through other means than inhibition of gibberellin biosynthesis, possibly by inducing ethylene synthesis [12].

Triarimol and other growth retardants are involved in membrane phenomena in both fungi and higher plants in which sterols are known to be membrane components [13, 14]. Also, triarimol seems to have an effect on higher plant hormones and hormones affecting cell expansion in fungi [13].

In *U. maydis*, triarimol has three sites of inhibition in the pathway or ergosterol biosynthesis: demethylation at C-14; introduction of the C-22(23) double bond; reduction of the C-24(28) double bond. The result of the inhibition is accumulation of three sterols in *U. maydis*: 24-methylenedihydrolanosterol, obtusifoliol and 14α-methyl-$\Delta^{8,24(28)}$ergostadienol. These three sterols largely replace ergosterol in triarimol treated cells. Triarimol strongly decreases the quantity of total sterol in *U. maydis* [2].

The work described here consists of experiments which were designed to determine the effect of triarimol on the growth, fatty acid and sterol composition of three species of *Chlorella*. It is important to study the effects of triarimol inhibition in a number of photosynthetic algae to determine if the lipid biosynthetic pathways are similar to those of heterotrophic organisms.

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RESULTS

Triarimol effect on Chlorella sorokiniana

Compared to previously studied sterol biosynthesis inhibitors in *Chlorella*, triarimol was not a strong inhibitor of the growth rate of *Chlorella sorokiniana*. The triarimol-treated cultures appeared darker green than control cultures. Although 2 mg/l. triarimol gave 50% inhibition of growth, further increases (up to 20 mg/l.) did not bring about significant increases in inhibition. A triarimol concentration of 10 mg./l caused a doubling of total chlorophyll compared to the control. These findings are consistent with the results of Shive [12] who showed that triarimol and ancymidol caused *Phaseolus vulgaris* leaves to be darker green than normal, and the results are also in accord with those of Leopold where ancymidol slightly increased the chlorophyll content of *Rumex* leaf discs [15].

An examination of the effect of triarimol on total lipids revealed that the amount of total lipid (% dry wt) was probably not significantly affected by triarimol except as it affected chlorophyll composition.

The triarimol treatment of *C. sorokiniana* produced an increase in the quantity of total fatty acids. A similar quantitative effect on fatty acids was observed in *Ustilago maydis* [2] and rats treated with triparanol [16].

The predominant fatty acids of triarimol treated *C. sorokiniana* were 16:0, 18:1, 18:2, 18:3. There was a sharp increase in triunsaturated (18:3) fatty acid from 0.2 to 16.0% of dry wt. In treated cells the ratio of unsaturated to saturated fatty acids was altered and contributed to an increase in total fatty acids (Table 1). A similar effect was observed in *Ustilago maydis* where there was an increase in the proportion of unsaturated free fatty acids [3]. The main unsaturated fatty acid which accumulated in treated *U. maydis* was linoleic acid [1]. There is evidence that a non-covalent complex between oleic acid and triparanol leads to annulment of toxicity in *Ochromonas* [17] and an annulment of toxicity of triarimol is accomplished by fatty acids (e.g. oleic acid) in *U. maydis* but not in *C. cucumerinum* [2]. These facts suggest that the increased concentration of fatty acids in treated cultures may produce a partial annulment of triarimol toxicity. This could help to explain why *C. sorokiniana* showed resistance to triarimol. A similar reversal of the toxic effects of triparanol in *Tetrahymena pyriformis* has been observed as a result of exogeneously added unsaturated fatty acids [18].

The effect of triarimol on sterol composition of Chlorella sorokiniana

The major sterols identified in control cultures of *C. sorokiniana* were identical to those described by Patterson *et al.* [5, 9, 19]. They were identified as 5α-ergost-7-en-3β-ol, ergosta-5,7-dien-3β-ol and ergosterol which was the dominant sterol and is considered to be the final sterol in the biosynthetic pathway of this species.

The digitonin-precipitated sterols from inhibited cultures were separated first by means of Al_2O_3 CC into 4-dimethyl, 4-monomethyl and 4-desmethyl sterols and identified by means of GLC [20] and by GC–MS [7, 8]. The 4-desmethyl fraction contained numerous sterols with overlapping peaks on GC. These sterols were separated by acetylation and chromatography on a $AgNO_3$–Si gel column and on a lipophilic Sephadex column [21].

Table 2 shows the sterol composition of *C. sorokiniana* in control and triarimol-treated cultures. The sterols identified in inhibited cultures have previously been isolated from triparanol-treated cultures of this organism [6]. The accumulation of significant quantities of 14α-methyl-9β,19-cyclo-5α-ergost-25-en-3β-ol and 4α,14α-dimethyl-5α-ergosta-8,25-dien-3β-ol indicates that triarimol was inhibiting the reduction of the Δ^{25} double bond. Sterols containing a methyl group at C-14 and at C-4 also accumulated. Accumulation of 14-methyl sterols as a result of the action of triparanol was first reported by Doyle *et al.* [7] and has been frequently noted in subsequent studies of other inhibitors [5, 19, 22].

The effect of triarimol on Chlorella ellipsoidea

Although 50% inhibition of growth of *C. ellipsoidea* was not reached until the triarimol concentration reached 10 mg/l., a further increase to 20 mg/l. resulted in a cessation of growth. At 10 mg/l. of triarimol, the chlorophyll concentration was higher than the control but it was not increased as much as in *C. sorokiniana*. Fatty acid content in *C. ellipsoidea* was apparently only slightly affected by triarimol (Table 1).

The effect of triarimol on sterol composition of Chlorella ellipsoidea

4-Dimethyl, 4-monomethyl and 4-desmethyl sterols were separated as with *C. sorokiniana*. Sterols from control cultures were identical to those described previously in *C. ellipsoidea* [5, 22] and were brassicasterol, ergost-5-en-3β-ol, poriferasterol, and clionasterol (Table 2). The sterol composition and quantity in *C. ellipsoidea* was markedly affected by triarimol. The sterols found in controls decreased to 41% of total sterols in triarimol-treated cultures. Poriferasterol was diminished by the largest amount.

Sterols accumulating in treated *C. ellipsoidea* (Table 2)

Table 1. Effect of triarimol on the relative proportions of various fatty acids in *Chlorella*

		Individual fatty acids as percentage of total fatty acids										Total* fatty acids
		16:0	16:1	16:2	16:3	16:4	18:0	18:1	18 2	18:3	18:4	
C. emersonii	Control	28.3	2.0	2.2	4.8	1.9	*t*†	31.0	5·9	17.0	2.1	3.9
	1 mg/l.	20.0	2.1	2.1	2.5	1.9	*t*	38.0	4.9	22.6	1.8	9.7
C. ellipsoidea	Control	20.8	—	*t*	—	—	*t*	33.6	25.2	14.6	—	9.6
	10 mg/l.	21.8	—	*t*	—	—	*t*	31.9	30.2	13.1	—	10.7
C. sorokiniana	Control	24.9	9.6	4.0	—	—	3.3	30.0	16 3	5.7	—	2.9
	2 mg/l.	14.9	5.7	5.4	—	—	*t*	11.2	12.3	45.9	—	33.3

* % dry wt.

† *t*—amount less than 1% of total fatty acids.

Table 2. Sterols of control and triarimol-treated* *Chlorella* sp.

Sterols†	*C. sorokiniana* Control	*C. sorokiniana* Treated	*C. ellipsoidea* Control	*C. ellipsoidea* Treated	*C. emersonii* Control	*C. emersonii* Treated
14α-Methyl-9β,19-cyclo-5α-ergost-25-en-3β-ol	—	7.0	—	—	—	—
Cyclolaudenol	—	0.4	—	—	—	—
Obtusifoliol	—	—	—	3.1	*t*	1.5
24-Dihydroobtusifoliol	—	—	—	21.8	1.0	5.2
4α,14α-Dimethyl-5α-ergosta-8,25-dien-3β-ol	—	6.0	—	—	—	—
4α,14α-Dimethyl-5α-(24*S*)-stigmast-8-en-3β-ol	—	—	—	8.7	—	3.0
24-Methylene pollinastanol	—	—	—	—	—	2.0
14α-Methyl-5α-ergost-8-en-3β-ol	—	3.7	—	15.3	1.7	15.9
14α-Methyl-5α-ergosta-8,25-dien-3β-ol	—	2.6	—	—	—	—
14α-Methyl-5α-(24*S*)-stigmast-8-en-3β-ol	—	—	—	9.7	—	3.4
5α-Ergosta-8(14),22-dien-3β-ol	—	2.6	—	—	—	—
5α-Ergost-7-en-3β-ol	13.3	8.1	—	—	15.2	12.5
5α-Ergosta-7,22-dien-3β-ol	—	2.7	—	—	*t*	0.3
Δ^7- Chondrillastenol	—	—	—	—	6.9	34.5
Chondrillasterol	—	—	—	—	75.2	17.0
Ergosta-5,7-dien-3β-ol	12.3	33.0	—	—	—	—
Ergosterol	74.4	29.3	—	—	—	—
Ergost-5-en-3β-ol	—	—	22.9	15.9	—	—
Poriferasterol	—	—	64.9	19.0	—	—
Clionasterol	—	—	6.7	6.0	—	—
Brassicasterol	—	—	5.5	*t*	—	—

* Triarimol: concentration: *C. sorokiniana* (6.0×10^{-6} M); *C. ellipsoidea* (3×10^{-5} M); *C. emersonii* (1.5×10^{-6} M).
† As % of total sterol.

frequently had the 14α-methyl group and the incidence of 22-double bonds was much less than in the control (Table 3). However, there was no accumulation of Δ^{25}-compounds as in *C. sorokiniana*. The ratio of sterols with 10 C side chains to those with 9 C side chains in inhibited cultures indicates that triarimol inhibited the second alkylation at C-24.

Looking at the frequency of different structures occurring in control and triarimol-treated *C. ellipsoidea* (Table 3), it can be observed that the ratio of sterols with 10 C side chains to those with 9 C side chains in control cultures was higher than in triarimol-treated cultures. The ratios were 2.5 and 0.8 respectively. This indicates that triarimol was inhibiting the second alkylation. These results are similar to those obtained by Chan [5] from triparanol-inhibited *C. ellipsoidea*. This inhibition occured in spite of a very low accumulation of obtusifoliol (3.0%) and lack of accumulation of other 24-methylene sterols. These sterols are considered as necessary intermediates for the second alkylation.

The effect of triarimol on Chlorella emersonii

Triarimol gave 50% inhibition of *C. emersonii* at 1 and 2 mg/l. prevented growth. Chlorophyll concentrations in *C. emersonii* were sharply reduced in inhibited cultures. The fatty acid composition of *C. emersonii* was increased by triarimol treatment with most of the increase accounted for by unsaturated fatty acids. The increase was not concentrated in linolenic acid as was the case in *C. sorokiniana* (Table 1).

The sterols of treated cultures fell into two groups (*a*) the Δ^7-sterols of control cultures and (*b*) 14α-methyl sterols. The second alkylation at C-24 was apparently

Table 3. Frequency of different sterol features occurring in control and triarimol-treated *Chlorella* sp.*

Sterol structure	*C. sorokiniana* Control	*C. sorokiniana* Treated	*C. ellipsoidea* Control	*C. ellipsoidea* Treated	*C. emersonii* Control	*C. emersonii* Treated
4,14-Dimethyl	—	6.4†	—	33.6	1.0	9.4
14α-Methyl Δ^8	—	12.7	—	58.6	2.8	27.9
9,19-Cyclopropane	—	7.4	—	—	—	1.9
$\Delta^{8,14}$	—	2.5	—	—	—	—
$\Delta^{8(9)}$ (desmethyl)	—	12.3	—	58.8	—	—
Δ^7	100.0	73.1	—	—	97.3	61.7
$\Delta^{5,7}$	86.7	62.3	—	—	—	—
Δ^5	86.7	62.3	100.0	30.9	—	—
Δ^{22}	74.4	34.6	70.5	19.0	75.2	16.6
Δ^{25}	—	16.0	—	—	—	—
24-Methylene	—	—	—	3.1	*t*	3.4
10 C Side chain	—	—	71.6	43.6	82.1	55.7

* Triarimol concentration: *C. sorokiniana*, 2 mg/l. (6×10^{-6} M); *C. ellipsoidea*, 10 mg/l. (3×10^{-5} M), *C. emersonii*, 1 mg/l. (3×10^{-6} M).
† As % of total sterol.

inhibited and the introduction of the Δ^{22} double bond was strongly inhibited.

DISCUSSION

Growth of 3 species of *Chlorella* indicated different levels of sensitivity to triarimol. *C. emersonii* was the most sensitive of these species although it was the least sensitive to triparanol and AY-9944 [8, 22].

Chlorophyll synthesis was inhibited almost completely in *C. emersonii* but was strongly stimulated in *C. sorokiniana* at 10 mg/l. triarimol concentration. *C. ellipsoidea* was affected less than *C. emersonii* by the exposure to triarimol, occupying an intermediary position among these 3 species with respect to chlorophyll content.

In all 3 species of *Chlorella*, triarimol treatment produced a buildup in the quantity of fatty acids. The unsaturated C-18 fatty acids, especially, increased in their amounts. The triunsaturated C-18 fatty acid in *C. sorokiniana* was increased by a large amount in treated culture.

Triarimol inhibited sterol biosynthesis in all 3 species. The most obvious effects were in the inhibition of the removal of the 14α-methyl and the inhibition of the introduction of the 22-double bond (effects which were seen in all 3 species). In *C. sorokiniana* there was an accumulation of Δ^{25}-sterols. In *C. ellipsoidea* it was shown that the 22-double bond cannot be synthesized from a saturated side chain [23]. Therefore, it is tempting to speculate that the pathway to the 22-double bond in *Chlorella* is $\Delta^{25} \rightarrow \Delta^{22,25} \rightarrow \Delta^{22}$. In this study triarimol inhibited the introduction of the 22-double bond (Table 3) and Δ^{25} sterols accumulate, as expected in such a pathway.

Further evidence of the effect of triarimol on the biosynthesis of sterol side chains is seen by comparing quantities of sterols with 10 C side chains and 9 C side chains in control and inhibited cultures. In both *C. emersonii* and *C. ellipsoidea* the second alkylation reaction at C-24 appeared to be inhibited. *C. sorokiniana* does not synthesize sterols with 10 C side chains.

EXPERIMENTAL

Chlorella emersonii Shihira and Krauss, Maryland Culture Collection No. 2 (Indiana Culture Collection No. 252), *Chlorella ellipsoidea* Gerneck, (Indiana Culture Collection No. 247), *Chlorella sorokiniana* Shihira and Krauss (Indiana Culture Collection No. 1230) were used.

Cultures were grown, harvested, and lipid extracted as described by Doyle [8]. Separation and identification of the fatty acids were achieved using GLC. The column used was 1.8 m × 3.4 mm and was packed with Gas Chrom P (Applied Science Labs) coated with 15% diethylene glycol succinate. Sterols were isolated by digitonin precipitation followed by chromatography on alumina to separate 4-dimethyl, 4-monomethyl and 4-desmethyl sterols. TLC on $AgNO_3$-impregnated Sil gel and chromatography on modified Sephadex LH-20 [21] was also used to separate the individual sterols. Sterols from all 3 species of *Chlorella* were analysed by GLC on 3% SE-30, 1% QF-1 and 3% HI-Eff-8BP. MS were obtained as described previously [7, 19]. MS of sterols identified were identical to those of known compounds.

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