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EFFECT OF TRIPARANOL AND 3β-(β-DIMETHYLAMINOETHOXY)-ANDROST-5-EN-17-ONE ON GROWTH AND NON-SAPONIFIABLE LIPIDS OF *SACCHAROMYCES CEREVISIAE**

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Abstract—Triparanol (8×10^{-5} M) completely suppressed the growth of *Saccharomyces cerevisiae*, whereas 3β-(β-dimethylaminoethoxy)-androst-5-en-17-one (10^{-4} M) displayed only slight growth-inhibitory properties. Preliminary observation suggests that major effects caused by triparanol on the incorporation of [1-^{14}C]sodium acetate into non-saponifiable lipids involved interference with the conversion of lanosterol to ergosterol, and to a lesser extent inhibition of squalene oxidation.

3β-(β-Dimethylaminoethoxy)-androst-5-en-17-one at 10^{-6} M caused no apparent reduction in the incorporation of [1-^{14}C]sodium acetate into the total non-saponifiable fraction, nor into ergosterol, yet did cause the accumulation of an unidentified compound. At a higher concentration (10^{-4} M) the major non-saponifiable compound that accumulated was isolated and characterized as 2,3-oxidosqualene. With prolonged incubation 2,3-oxidosqualene appeared to be converted to one or more compounds less polar than lanosterol.

Triparanol and 3β-(β-dimethylaminoethoxy)-androst-5-en-17-one (DMAE-DHA) are two examples of hypocholesterolemic compounds containing a dimethylaminoethoxy group. Both lower serum cholesterol levels and have been shown to inhibit a step in the cholesterol biosynthetic pathway-reduction of desmosterol to cholesterol [1, 2].

The effects of both compounds on lipid metabolism in other biological systems have also been studied. Triparanol causes growth inhibition of the protozoan *Tetrahymena pyriformis* [3], and has been shown to inhibit the conversion of squalene to tetrahymanol by this organism [4, 5]. It has also been suggested that it interferes with the synthesis of unsaturated fatty acids by *T. pyriformis* [3, 6] and also by *Ochromonas danica* [7]. Malhotra and Nes [8] have demonstrated that triparanol inhibits the C-24 alkylation of sterols in a cell-free system from germinating peas, whereas in *Chlorella* [9, 10] triparanol appears to inhibit other reactions of sterol biosynthesis; viz removal of the 14α-methyl group, the second alkylation at C-24, reduction of C-7, and the Δ^8-Δ^7 isomerization.

The effects of DMAE-DHA on growth and on several areas of metabolism of *T. pyriformis* have been studied. Inhibition of the conversion of squalene to tetrahymanol [11] and to diplopterol [12] appeared to be the proximate cause of growth inhibition. Sipe and Holmlund [5] noted inhibition by DMAE-DHA of both cyclization of 2,3-oxidosqualene and conversion of C_{30} to C_{27} sterols by rat liver homogenates.

Although inhibition of yeast growth by triparanol has been reported by Aaronson [13], no data have appeared concerning the effects of this compound on lipid metabolism by this organism. This paper will present initial findings concerning the effects of triparanol and DMAE-DHA on the growth of *S. cerevisiae* and on the conversion of [1-^{14}C]sodium acetate to nonsaponifiable lipids by this organism.

MATERIALS AND METHODS

Growth and maintenance of S. cerevisiae. *S. cerevisiae*, strain MY306, was obtained through the courtesy of Dr. Eugene Dulaney, Research Laboratories Merck & Co., Inc. Rahway, N.J., and was maintained and grown on a medium consisting of Difco yeast extract (2%), ammonium chloride (0.1%), K_2HPO_4 (1.1%), KH_2PO_4 (1.85%) and glucose (2%). Cultures were incubated at 28° in a Psychrotherm incubator (New Brunswick Scientific Co., New Brunswick, N.J.) with shaking at 50 rpm. Cultures in 18 × 150 mm tubes were incubated at approximately 60° from the vertical to improve aeration. Seventeen-hour cultures were employed as inoculum (5%, v/v) to initiate experiments. For experiments involving growth studies and [1-^{14}C]sodium acetate incorporation a freshly inoculated culture was added to sterile tubes or flasks containing triparanol or DMAE-DHA, which were added in methanol followed by removal of solvent *in vacuo*. Growth was followed by measurements of the absorbance of the culture at 640 nm (A_{640}) with a Bausch and Lomb Spectronic 20, Uninoculated medium, suitably diluted, was used as a blank. All results are presented as mean values of duplicate test samples. Agreement between duplicates was within 10 per cent.

Extraction and analysis of lipids. Cells were centrifuged at 1000 *g* for 10 min. in a Sorvall RC-2B centri-

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B.P. 25/11—B

fuge, washed twice with distilled water (0.5 × the original volume of the culture), and extracted three times with 0.2 vol of methanol. This procedure yielded reproducible results, provided more ^{14}C-non-saponifiable lipids than could be obtained from cells disrupted by one passage through a French press, followed by lyophilization and extraction with chloroform-methanol (2:1; v/v), and yielded about 90 per cent of the ^{14}C-lipids extractable by the more cumbersome Deierkauf and Booij procedure [14].

Saponification was conducted by addition of sufficient 10% aqueous potassium hydroxide (w/v) so that the final volume ratio of methanol to water was 3:2. The solution was allowed to stand overnight at room temperature. This procedure effected hydrolysis of triglycerides and phospholipids, but was without significant effect on steryl esters.

^{14}C-Lipids were extracted from the saponification mixture with petroleum ether and were resolved by thin-layer chromatography (t.l.c.) on Silica gel G plastic plates (Brinkman Instruments, Inc., Westbury, N.Y.) by developing twice with benzene–ethyl acetate (5:1; v/v) to 10 cm and once with petroleum ether–diethyl ether–glacial acetic acid (95:5:1, v/v) to 15 cm. Reference compounds were visualized by brief exposure to iodine, and the ^{14}C-labeled lipids were detected by radioautography [11]. ^{14}C-Labeled zones were cut from the plate, added to scintillation vials, and radioactivity was measured in a Packard Tricarb Scintillation Spectrometer after addition of 5 ml of scintillation fluid consisting of 4 g 2,5-diphenyloxazole, 0.1 g bis-*o*-methylstyryl(-benzene), 660 ml toluene and 340 ml cellosolve.

Analytical gas liquid chromatography (g.l.c.) was conducted with a model 5750 gas chromatograph (Hewlett Packard Instrument Co., Avondale, PA.) equipped with a flame ionization detector. A 6-foot, 1/8-inch i.d. stainless steel column packed with Anachrom ABS (90/100 mesh) impregnated with 1% SE30 was employed. The oven temperature was 220° and the nitrogen carrier gas flow was 25 ml/min. Preparative g.l.c. was carried out with a Varian Aerograph Gas Chromatograph model 90-P. The carrier gas used was helium (40 ml/min). A 5-foot, 1/4-inch i.d. column packed with Anachrom ABS (90/100 mesh) and impregnated with 1% SE30 was employed. The column temperature was 220° and the detector temperature 300°. The collector port was heated to about 157° by a nichrome wire to prevent sample condensation in the system. As the recorder revealed the emergence of a fraction from the column a collector tube, immersed in a dry ice–acetone bath, was connected to the collector outlet.

I.r. spectra were taken with a model IR-8 infrared spectrophotometer (Beckman Instruments, Fullerton, CA). Preparations were dissolved in carbon tetrachloride. Mass spectra were obtained with a DuPont model 21-491 mass spectrometer under the following operating conditions: source temperature 250°; inlet probe temperature 100°; source pressure 4×10^{-7} torr; 70 eV; filament current, 300 microamps.

Chemicals. DMAE-DHA was kindly provided by Dr. S. Bernstein, Lederle Laboratories, Pearl River, N.Y. Triparanol is a product of the William S. Merril, Co., and was kindly supplied by Dr. Glenn Patterson, Department of Botany, University of Maryland. [1-^{14}C]Sodium acetate was obtained from New England Nuclear, Boston, MA. Yeast extract was purchased from Difco Laboratories, Detroit, MI. Reference Compounds for t.l.c. were purchased from Applied Science Laboratories, Inc., State College, PA. 2,3-Oxidosqualene was kindly provided by Dr. J. D. Sipe.

Solvents were redistilled before use; chloroform 59–61°, petroleum ether 68–70° and diethyl ether 34–36°. All other solvents and chemicals were reagent grade.

RESULTS AND DISCUSSION

Effects on growth of S. cerevisiae. From the data of Table 1 it appears that triparanol is a more active inhibitor of the growth of *S. cerevisiae* than is DMAE-DHA. At 8×10^{-5} M triparanol completely suppressed growth, whereas DMAE-DHA at 10^{-4} M had little effect on the growth rate. Even at 10^{-3} M DMAE-DHA succeeded only in sharply curtailing the growth rate without totally abolishing cellular multiplication. In contrast, DMAE-DHA was somewhat more active than triparanol against the protozoan *T. pyriformis* [5].

Effects on incorporation of [1-^{14}C]*sodium acetate into non-saponifiable lipids.* Table 2 presents data on the effects of several concentrations of triparanol and DMAE-DHA on the incorporation of [1-^{14}C]sodium acetate into non-saponifiable lipids. As indicated in

Table 1. Effects of triparanol and DMAE-DHA on growth of *S. cerevisiae*

Compound	Concentration M	A_{640} 2 hr	4 hr	6 hr	8 hr	12 hr
Triparanol*	0	—	2.3	—	4.5	5.4
	2×10^{-5}	—	1.6	—	3.2	4.9
	8×10^{-5}	—	0.41	—	0.36	0.32
DMAE-DHA†	0	0.58	2.8	5.7	—	5.5
	10^{-4}	0.58	2.3	4.0	—	5.5
	10^{-3}	0.47	0.56	0.74	—	2.5

* Fifty ml. of culture, initial $A_{640} = 0.26$, were added to sterile 500-ml Erlenmeyer flasks containing sufficient triparanol as a dry residue to provide the indicated final concentration.

† Five ml of culture, initial $A_{640} = 0.35$, were added to sterile 18 × 150-mm culture tubes containing sufficient DMAE-DHA as a dry residue to provide the indicated final concentration.

Table 2. Effects of triparanol and DMAE-DHA on production of ^{14}C-non-saponifiable lipids from [1-^{14}C]sodium acetate by *S. cerevisiae*

Compound	Concentration M	Non-saponifiable lipids* 4 hr	8 hr	24 hr
Triparanol*	0	19.1 (2.3)	25.2 (4.5)	—
	2×10^{-5}	8.6 (1.6)	17.6 (3.7)	—
	5×10^{-5}	3.1 (0.43)	3.9 (0.46)	—
	8×10^{-5}	3.2 (0.41)	3.7 (0.36)	—
DMAE-DHA†	0	25.0 (2.7)	—	36.2 (11.0)
	10^{-6}	33.4 (2.7)	—	43.0 (11.5)
	10^{-5}	27.2 (2.7)	—	38.6 (11.5)
	10^{-4}	32.8 (2.3)	—	44.6 (10.5)

* Data are expressed as cpm $\times 10^{-4}$ per 5 ml culture. Values in parentheses represent A_{640} turbidity readings—a measure of cell population density.

† The experiment was initiated as described for triparanol in Table 1. An ethanol solution (0.8 ml, 800 μCi) of [1-^{14}C]sodium acetate (59 mCi/mmole) was added to 400 ml of culture before distribution of 50-ml aliquots to flasks for incubation. Ten-ml aliquots of culture were removed at intervals for extraction and analysis of lipids.

the Material and Methods section the saponification conditions employed were without effect on steryl esters, which continue therefore to be present in the 'non-saponifiable lipids' fraction. Commensurate with its more drastic effects on growth, triparanol, even at 2×10^{-5} M caused a sharp reduction in the rate of incorporation of the label into the non-saponifiable lipid fraction. DMAE-DHA, at concentrations up to 10^{-4} M, exerted no inhibition on the rate of formation of labeled non-saponifiable lipids.

Of considerable interest is the relative distribution of label in the non-saponifiable fraction from yeast grown in the presence of triparanol or DMAE-DHA. Figures 1 and 2 are radioautographs of non-saponifiable lipid fractions, resolved by t.l.c., from cultures exposed to triparanol and [1-^{14}C]sodium acetate for 4 and 8 hr. Visual inspection of Figure 2 suggests that at 2×10^{-5} M triparanol might cause an accumulation of a substance with t.l.c. mobility comparable to that of lanosterol (zone D). At higher concentrations of triparanol this accumulation is more pronounced, and the 'ergosterol' zone is only slightly labeled in the presence of $5–8 \times 10^{-5}$ M triparanol. Moreover there appears to be a greater accumulation of a substance (zone K) with mobility characteristic of squalene than witnessed in the controls. Zones B ('ergosterol'), D ('Lanosterol'), J ('steryl esters') and K ('squalene') were removed from the t.l.c. plate for determination of the ^{14}C content. Analysis of the 4-hr samples (Fig. 1) revealed that in the presence of

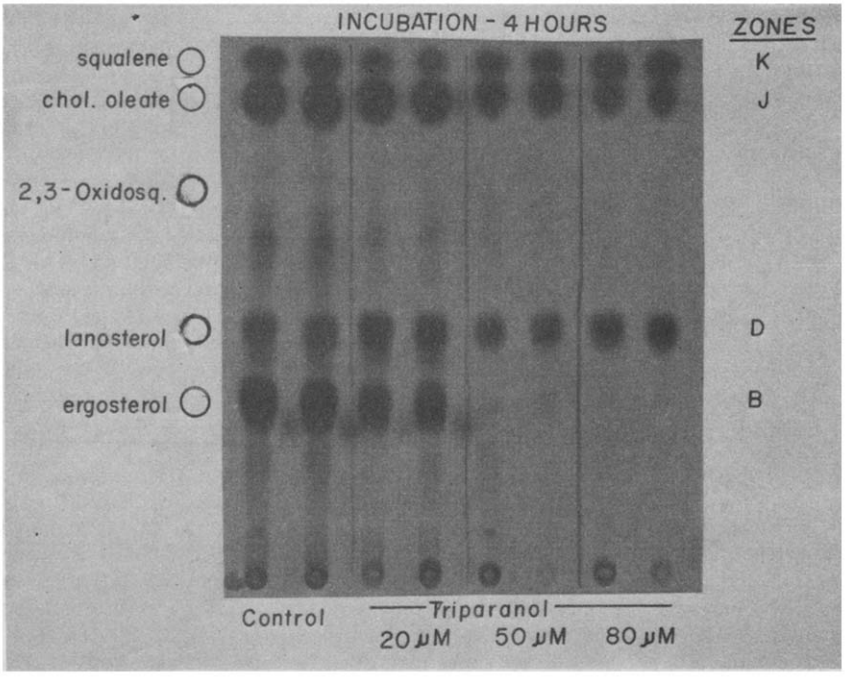


Fig. 1. Effect of triparanol on incorporation of [1-^{14}C]sodium acetate by *S. cerevisiae* into non-saponifiable lipids. Cultures were exposed to [1-^{14}C]sodium acetate and varying concentrations of triparanol for 4 hr before extraction and analysis by t.l.c. of the non-saponifiable fractions.

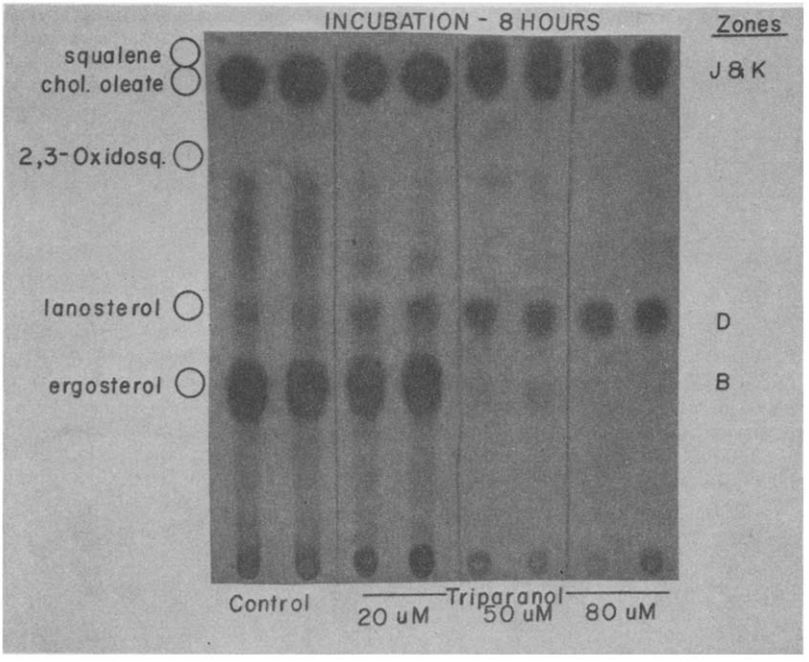


Fig. 2. Effect of triparanol on incorporation of [1-^{14}C]sodium acetate by *S. cerevisiae* into non-saponifiable lipids. Cultures were exposed to [1-^{14}C]sodium acetate and varying concentrations of triparanol for 8 hr before extraction and analysis by t.l.c. of the non-saponifiable fraction.

2×10^{-5} M triparanol, the incorporation of ^{14}C into 'ergosterol' represented 26 per cent of the total non-saponifiable lipids, whereas for the controls the value was 37 per cent. Comparable values for the 'lanosterol' zone were 6.5 per cent for the control and 15.8 per cent for the samples incubated with 2×10^{-5} M triparanol. The fraction of ^{14}C-non-saponifiable lipids represented by 'ergosterol' and 'lanosterol' for samples incubated with 5×10^{-5} M triparanol were 1.9 per cent and 25 per cent, respectively. The steryl ester fraction represented 29 per cent of the non-saponifiable lipids for the control and this fraction was, surprisingly, not greatly affected by triparanol at $2–5 \times 10^{-5}$ M. The fraction of non-saponifiable lipids represented by 'squalene' was almost two to three times control values in the $5–8 \times 10^{-5}$ M samples. Analysis of the 8-hr incubation data (Fig. 2) revealed similar trends.

These observations, though admittedly premature because of incomplete characterization of the labeled fractions, do suggest that in yeast triparanol affects the metabolism of non-saponifiable lipids primarily at one or more sites subsequent to the formation of lanosterol, and secondarily restricts the rate of epoxidation of squalene. Similar findings have been made in a rat liver system [15]. Major effects of triparanol on events subsequent to cyclization of 2,3-oxidosqualene have also been reported for a cell-free system from germinating peas [8] and with intact *Chlorella* species [9, 10].

Figures 3 and 4 represent radioautographs of ^{14}C-labeled non-saponifiable lipids from yeast incubated for 4 and 24 hr with varying concentrations of DMAE-DHA. Most startling perhaps is the observation that at 10^{-6} M DMAE-DHA, a concentration only 1 per cent of that causing only slight growth inhibition, there was a significant modification in the pattern of non-saponifiable lipids. By 24 hr (Fig. 4) substances in zone G are significantly labeled (about 7.9 per cent and 5.3 per cent of total non-saponifiable lipids) in samples from cultures incubated with 10^{-5} M and 10^{-6} M DMAE-DHA, respectively. In contrast, radioactivity from the same zone from controls represented only 0.8 per cent of the non-saponifiable lipids. Exposure to 10^{-5} M DMAE-DHA revealed by 4 hr (Fig. 3) additional abberations from control data. When expressed as a percent of the non-saponifiable lipids comparisons with the control were as follows: 'ergosterol' was 20.9 (31.0 for the control); 'lanosterol' was 28.3 (5.7 for the control); zone F was 2.9 (1.2 for the control) and zone H was 8.2 (0.6 for the control). 'Steryl esters', though not quantitatively measured, also appeared to be reduced in quantity. At the highest concentration of DMAE-DHA tested, 10^{-4} M, there appeared to be no label in the 'steryl ester' zone; 'ergosterol' represented only about 3 per cent of the total non-saponifiable lipids, whereas zone H amounted to 54 per cent. There appeared to be a gradual movement of ^{14}C from zone H to zone F with time (cf Figs. 3 and 4) when cultures were incubated with 10^{-5} to 10^{-4} M DMAE-DHA.

As will be described, the substance in zone H has been characterized as 2,3-oxidosqualene. One possible explanation for the observations made with differing concentrations of DMAE-DHA is suggested in the following scheme where the indicated concentration of DMAE-DHA causes a partial or complete blocking of the reaction.

At the highest concentration of DMAE-DHA, 10^{-4} M, formation of lanosterol from 2,3-oxidosqualene is almost completely blocked. However, with increasing time of incubation 2,3-oxidosqualene is con-

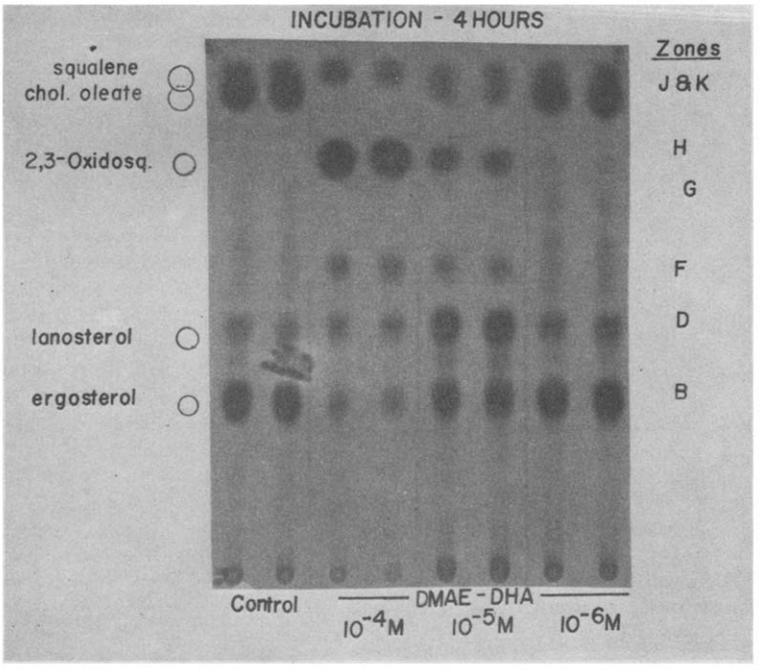


Fig. 3. Effect of DMAE-DHA on incorporation of [1-^{14}C]sodium acetate by *S. cerevisiae* into non-saponifiable lipids. Cultures were exposed to [1-^{14}C]sodium acetate and varying concentration of DMAE-DHA for 4 hr before extraction and analysis by t.l.c. of the non-saponifiable fraction.

sumed and apparently converted to F. At 10^{-5} M DMAE-DHA there is an early accumulation of 2,3-oxidosqualene and lanosterol together with a smaller quantity of F. By 24 hr the 2,3-oxidosqualene and lanosterol have disappeared some F is still present, and G has been formed. Steryl esters are less abundant than in the control, while the ergosterol level is comparable to the control. Since F appears to come from 2,3-oxidosqualene, and F and G are observed in significant quantity only in the presence of DMAE-DHA, it is possible that F and G are intermediates in the biosynthesis of ergosterol or of steryl esters. As depicted in the scheme, either F or lanosterol might serve as a precursor for G. Isolation and

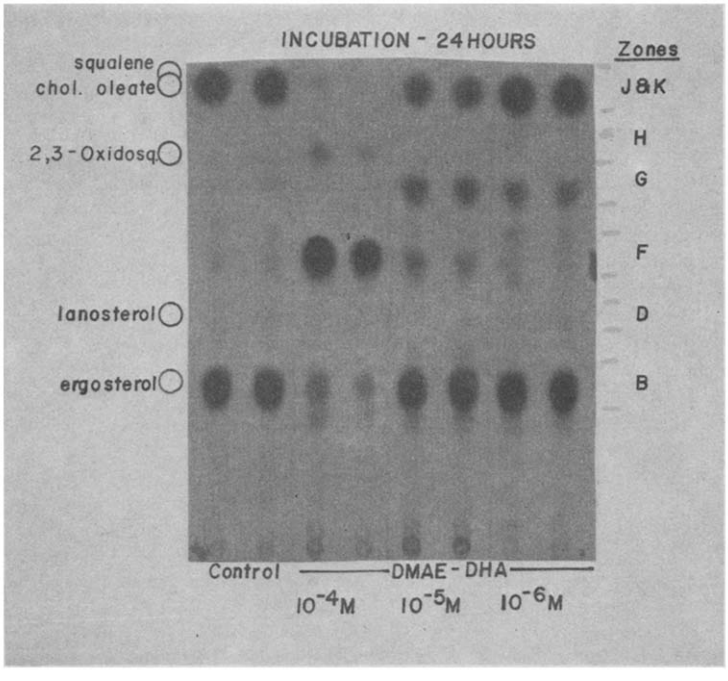
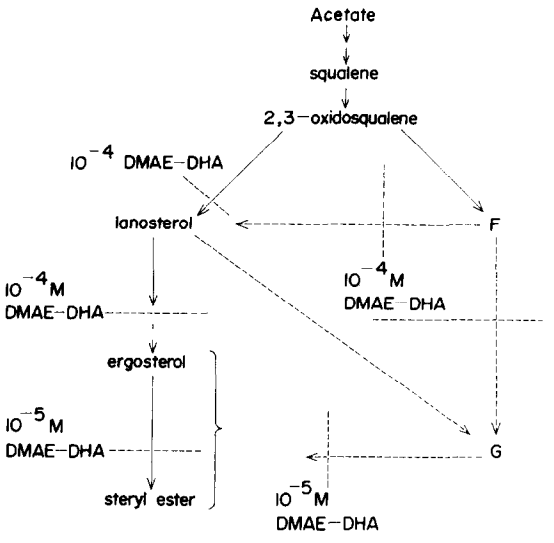


Fig. 4. Effect of DMAE-DHA on incorporation of [1-^{14}C]sodium acetate by *S. cerevisiae* into non-saponifiable lipids. Cultures were exposed to [1-^{14}C]sodium acetate and varying concentrations of DMAE-DHA for 24 hr before extraction and analysis by t.l.c. of the non-saponifiable fraction.



identification of F and G will be required to elucidate their role in the biosynthesis of sterols and steryl esters.

Identification of zone H substance as 2,3-oxidosqualene. Two Fernback flasks, each containing one liter of sterile medium with DMAE-DHA (10^{-4} M), were inoculated with 50-ml portions of an overnight culture of *S. cerevisiae*. After 4 hr incubation lipids were extracted from the culture and saponified. The non-saponifiable fraction weighed approximately 30 mg. By t.l.c. it was found that the most abundant component in the non-saponifiable preparation was zone H material with a small quantity of zone K material. After concentration the entire non-saponifiable fraction was chromatographed on a thick layer plate using the same chromatographic conditions described in the Materials and Methods section. The substance migrating in zone H was recovered by scraping the plate and extracting three times with 15-ml portions of methanol. The extracts were combined, filtered, and concentrated under nitrogen to about 3 ml. Aliquots were analyzed by t.l.c. employing three different developing systems. In each case only one substance was observed, and it migrated in the same manner as 2,3-oxidosqualene. The R_f values were as follows: 0.58 for the developing conditions described in the Materials and Methods section, 0.26 for the system consisting of petroleum ether–ethyl acetate (95:5, v/v), and R_f 0.39 with methylene chloride.

I.r. spectra for the zone H substance and for authentic 2,3-oxidosqualene were obtained. The spectra were very similar, and both exhibited maxima at 2960, 2920, 2860, 1445, 1375 and 1245 cm^{-1}. These maxima agree with those reported by Willet *et al.* [16] for 2,3-oxidosqualene.

Further evidence for the purity and identity of zone H substances as 2,3-oxidosqualene was obtained by g.l.c. and mass spectroscopy. Analytical g.l.c. on a 1% SE30 column at 220° indicated a relative retention time of 1.88 compared to squalene for each of the two preparations. Preparative g.l.c. was also conducted with a 1% SE30 column. Three fractions were collected from the peak emerging as 2,3-oxidosqualene from the authentic compound and from the zone H substance. All were analyzed by mass spectroscopy and were found to display mass spectra with the following *m/e* values: 426, molecular ion, 357, 203, 191, 189, 177, 175, 163, 161, 153, 149, 147, 137, 135, 123, 121, 109 and 107. Willet *et al.* [16] reported the same *m/e* values for 2,3-oxidosqualene.

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