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Effect of Triparanol on Synthesis of Squalene and Tetrahymanol by *Tetrahymena pyriformis*.* (30065)

MARY S. SHORB, BRIAN E. DUNLAP AND WILLIAM O. POLLARD†
Poultry Science Department, University of Maryland, College Park, Md.

Triparanol, an inhibitor of cholesterol synthesis in animals, is also an effective inhibitor of growth(1-5) and fatty acid synthesis (4,5) in *Tetrahymena* species. While certain specific sterols and fatty acids reversed the growth-inhibiting effect of triparanol citrate on *T. pyriformis* mating type II, variety 1 (6) a study of the sterols in the inhibited cultures was not made. Pollard *et al*(4) had observed shifts in the concentration of unidentified steroid substances from inhibited *T. pyriformis* W. Shorb(7,8) and Pollard *et al*(4,5) also observed the effect of other growth supplements, acetate, propionate, isobutyrate and α-methyl-*n*-butyrate, on lipid synthesis. The present paper describes studies on the effect of triparanol on some non-saponifiable components of *T. pyriformis*. It also includes a description of some properties of the triterpenoid alcohol, tetrahymanol, the principal component in the non-saponifiable fraction of *Tetrahymena*(9).

Experimental. Stock cultures of *T. pyriformis* were maintained on 1% yeast extract broth containing 10 mg/ml of glucose, sterilized separately and added asceptically. For studies on the isolation and properties of tetrahymanol, cultures of *T. pyriformis* W were grown 5 days in 2 liter batches of proteose peptone broth(10). In one experiment with proteose peptone medium, triparanol citrate was added at 1 μg/ml. For all other triparanol or triparanol citrate inhibition studies, the synthetic medium, growth supplements and the cultivation methods for the W, H and S strains of *T. pyriformis* were the same as those used by Pollard *et al*(5). Other cultural conditions are mentioned in each experiment.

Total lipids were extracted from centrifuged, frozen and thawed cells with chloroform-methanol (2:1, v/v). One aliquot was methylated with BF_3 methylating reagent.‡ This fraction, designated "methylated total lipids," was further partitioned into steroid material and fatty acid methyl esters on a silicic acid column(11), the fatty acid methyl esters and squalene being removed with benzene-hexane solvent (1:1, v/v). The steroid material was eluted with chloroform, followed by methanol. A second aliquot of total lipid was separated on a silicic acid column using chloroform to elute the neutral lipids and methanol to elute the phospholipids(11).

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† Present address: School of Pharmacy, Duquesne University, Pittsburgh, Pa.

‡ Applied Science Lab., State College, Pa.

Components of these fractions were further separated and identified by thin layer chromatographs (TLC) and by gas-liquid chromatography (GLC) as described below.

TLC plates of silica gel G§ were developed in hexane-ethyl ether-acetic acid, 90:10:1, v/v (solvent I) or cyclohexane-benzene, 4:1, v/v (solvent II). The plates were pre-run in the respective solvent. Samples were spotted or streaked on the plate and the plates developed in a saturated chamber usually to a predetermined solvent front. Appropriate pure compounds were run as standards. Lipid spots were detected by exposure to iodine vapors; by spraying with 0.2% 2′,7′-dichlorofluorescein in 95% ethanol followed by exposure to ultraviolet light; or by spraying with 5% phosphomolybdic acid in 95% ethanol, followed by heating for 10 minutes at 100°C. The latter spots were marked and the plates reheated to 200°C to bring out spots (tetrahymanol) not developed at 100°C or by iodine vapors. Non-saponifiable components were analyzed by GLC on a Barber-Colman instrument equipped with a radium detector, using argon gas carrier. Columns were packed with 1% G_1 SE 30 or 1% G_{19} Fluorosilicone QF-1-0065 (QF_1) on Anakrom ABS 90/100 mesh.‖ Operating conditions are stated with each experiment.

Tetrahymanol, the compound named by Mallory *et al*(9) but described by McKee *et al*(10) as isomeric with cholesterol, was isolated in two ways. By McKee's method, which is essentially a saponification of an acetone-ethyl ether extract, tetrahymanol was precipitated with ethanol as a white material from a yellow oil. We purified it further by chromatographing twice on TLC, using solvent system I. In the second method, frozen and thawed cells were saponified with 20% KOH in 40% ethanol for 2 hours on a steam bath. This mixture was extracted with hexane, followed by chloroform. The 2 non-saponifiable fractions were combined, washed with distilled water, dried with a rotary vacuum and purified by TLC as described above. This preparation of tetrahymanol was as pure as that made by the McKee method.

§ E. Merck AG, Darmstadt, Germany.

‖ Analabs, Hamden, Conn.

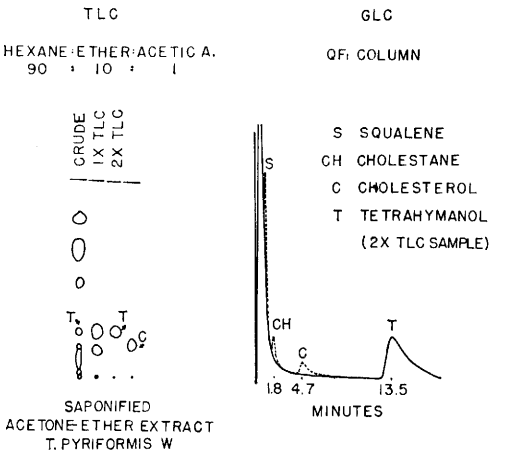

FIG. 1. Left hand side shows tetrahymanol (T) purification on thin-layer silica gel G plates, from saponified acetone-ether extracts of *T. pyriformis* W(10). Right hand side shows gas-liquid chromatograms of the 2 times purified tetrahymanol on QF_1 column at 222°C, 18 lb inlet pressure compared with known pure compounds (dotted lines).

Its properties differed from cholesterol on TLC, GLC, infra red spectrum and in other tests.

On TLC, tetrahymanol (zone T, Fig. 1, left side) traveled ahead (R_f 0.14) of cholesterol (R_f 0.10) and behind free fatty acids. Tetrahymanol stained very faintly with iodine vapors, whereas cholesterol stained strongly. Cholesterol produced a dark blue spot with phosphomolybdic acid spray after heating the plate to 100°C; good color developed with tetrahymanol only after heating for about 10 minutes at 200°C. As little as 0.1 μg of tetrahymanol could be detected faintly on TLC plates while 0.3 to 1 μg was seen readily, especially on plates freshly stained with phosphomolybdic acid.

The properties of tetrahymanol on GLC are also quite different from those of cholesterol. Purified tetrahymanol from TLC, zone T, Fig. 1, had a relative retention time (RRT) of 7.4 (see peak T,¶ right hand side) compared with cholestane, 1.0, and cholesterol, 2.6, when run on the QF_1 column using conditions stated under Fig. 1. On an SE 30

¶ Although authentic samples of tetrahymanol have not been available, Dr. R. L. Connor, Dept. of Biology, Bryn Mawr College, Bryn Mawr, Pa. in a personal communication, confirmed our observation that peak T was tetrahymanol.

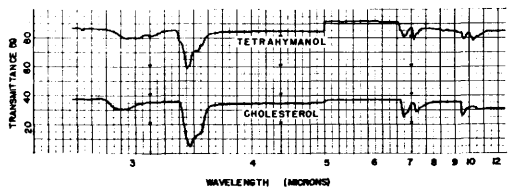

FIG. 2. Infra-red spectrum of tetrahymanol and cholesterol.

column, at 225°C and 15 lb inlet pressure, the RRT of cholesterol was 2.02 and tetrahymanol, 5.04. The infra red reflectance spectrum** of tetrahymanol (Fig. 2) also showed slight differences from cholesterol at wavelengths 2.85 to 3.2; 3.3 to 3.6; 6.3 to 7.3; at 8.5 and 9.2 to 11 μ.

A few miscellaneous observations were made on tetrahymanol. It is very stable, having been found in lipid samples stored for at least 4 years. It is found free in the neutral lipid fraction of the total lipid extracts; ester forms were not observed. Tetrahymanol occurs in low amounts in the total lipids, twice TLC-purified tetrahymanol representing about 0.4% of the original total lipid, and, after a second precipitation from absolute ethanol, about 0.07% of the original lipid. Connor¶ observed that at least 95% of the non-saponifiable fraction of *T. pyriformis* was tetrahymanol.

Having identified tetrahymanol on TLC and GLC, and studied some of its properties, it was of interest to investigate the effect of triparanol on the synthesis of tetrahymanol and other non-saponifiables.

Results. Fig. 3 shows tracings of thin layer chromatograms of total lipids of cultures of *T. pyriformis* W grown with (0.6 μg/ml) and without triparanol citrate (left hand column) or grown 6 to 8 days supplemented with 0.4 mg/ml of α-methyl-*n*-butyrate (right hand column). Lipid spots were developed with iodine and 2′,7′-dichlorofluorescein. One conspicuous spot, having an Rf of 0.9-1.0 on various runs, appeared just below the solvent front in triparanol-inhibited cultures. This fast-moving spot also appeared on TLC of the total lipids of triparanol-inhibited cultures of strains S and H containing α-methyl-*n*-butyrate supplement (0.5 mg/ml, data not shown). This spot moved just ahead of the standard cholesterol oleate and was not seen in any uninhibited culture at the concentrations chromatographed.

Fig. 4 shows superimposed chromatograms of "methylated total lipids" of other cultures of *T. pyriformis* grown with and without triparanol (1 μg/ml of culture) and containing acetate, propionate or α-methyl-*n*-butyrate supplements(5), chromatographed on GLC SE 30 column. One substance, with a peak at about 4.8 minutes, appeared in greater concentration in the lipids from inhibited cultures (broken line) than in the uninhibited cultures and had a relative retention time which corresponded to that of squalene. Other peaks at about 6.5, 11.0 and 13 minutes also increased while the one between 22 to 25

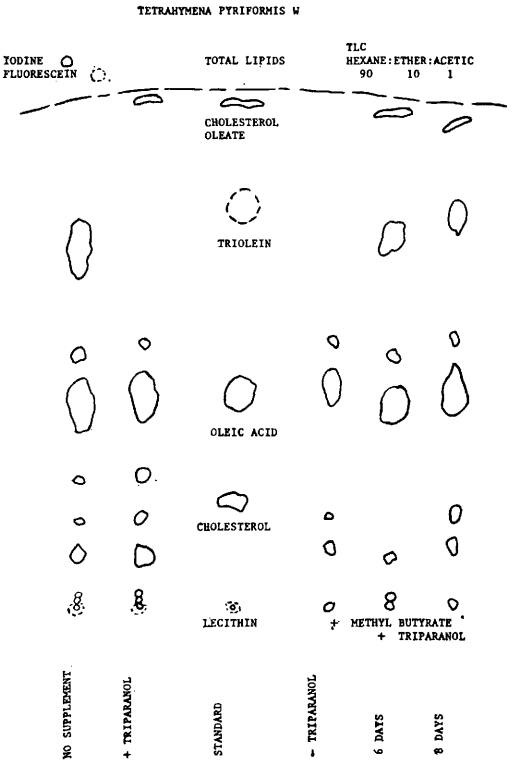

FIG. 3. Tracings of thin-layer chromatograms of total lipids of *T. pyriformis* W grown for 6 and 8 days with and without triparanol citrate; with α-methyl-*n*-butyrate supplement, right-hand side.

** IR spectrum was taken with Model 12 internal reflectance attachment (Wilkes Scientific Corp., South Norwalk, Conn.) with KRS-5 crystal, run on a Perkin-Elmer Model 421 IR spectrophotometer. We are indebted to Miss Marjorie S. Malmberg, Chemistry Dept., Univ. of Maryland, for the IR analyses.

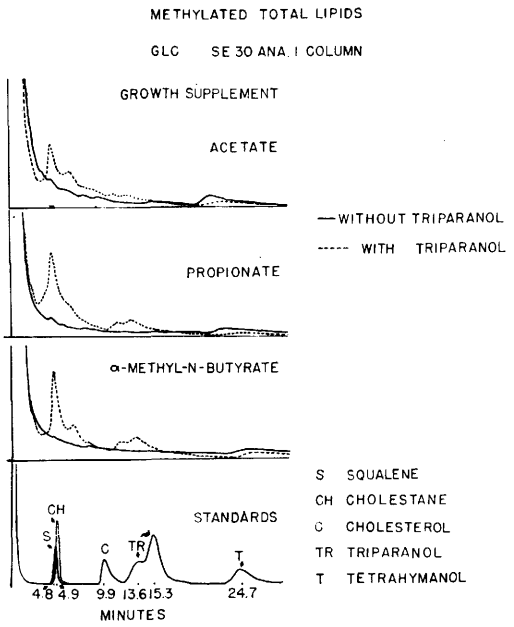

FIG. 4. GLC tracings of methylated total lipids from *T. pyriformis* W grown with and without triparanol in acetate, propionate and α-methyl-*n*-butyrate supplemented medium; column at 225°C with 15 lb inlet pressure.

minutes decreased. The unidentified lipid peaks at 11 and 13 minutes might possibly be triparanol, as triparanol treated with BF_3 methylating reagent produced peaks with similar retention time and appearance. Non-methylated triparanol had a double peak at 13.6 and 15.3 minutes (Fig. 4, bottom). The peak at 22 to 25 minutes was identified as tetrahymanol by comparison with a purified tetrahymanol standard (bottom tracing). The peaks representing tetrahymanol and triparanol emerged slightly earlier than those of the standards in separate analyses. No peak corresponding to cholesterol has been found in this or other chromatograms. The peak at 6.5 minutes and other smaller peaks from inhibited cultures were not identified.

To establish the common identity of the 4.8 minute GLC peak (Fig. 4) and the fast-moving TLC component, untreated total lipids were streaked on a TLC plate and chromatographed in solvent I. The component with an R_f of 0.9-1.0 was scraped off, eluted with benzene, dried and run on GLC, under conditions used in Fig. 4. A peak emerged at 4.8 minutes. This material, rechromatographed on TLC with hexane as solvent, had an R_f of 0.15, as did squalene. This twice purified sample was injected with pure squalene on GLC and only one peak emerged at 4.8 minutes. Furthermore, when the "methylated total lipids" were fractionated on a silicic acid column into steroid material and methyl esters of fatty acids, the latter fraction contained a peak emerging at 4.8 minutes, identical with squalene on the SE 30 GLC column. Pure squalene, added to "methylated total lipids" and subjected to the same silicic acid column fractionation, separated with the fatty acid methyl esters. This fact was not recognized in a preliminary report(4), which stated that squalene was not identified, because the fatty acid methyl ester fraction had not been examined on the SE 30 column, usually used only for steroid materials.

Further evidence that the fast-moving TLC fraction was identical with squalene was demonstrated by subjecting the twice-purified TLC material to infra-red analysis. The tracings of this and pure squalene are shown in Fig. 5. Except for variations due to quantities, the tracings are identical. Also, the melting point of the bromide of the isolated fast-moving material was the same as that of pure squalene and the ultraviolet spectrum was identical. On 2-way TLC, using solvents I and II, the unknown and squalene had identical R_f values.

To establish the extent to which squalene was increased and tetrahymanol or other sterols were decreased in triparanol-inhibited cultures, *T. pyriformis* W strain was grown for 5 days in proteose peptone medium with

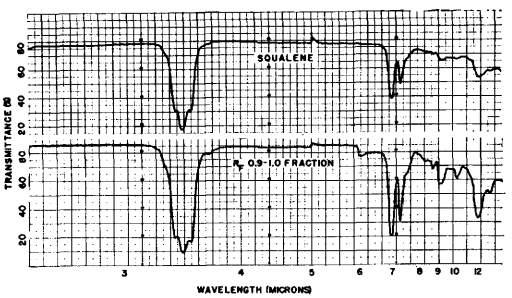

FIG. 5. Infra-red spectrum of squalene and R_f 0.9-1.0 fraction.

TABLE I. Per Cent Composition of Non-Saponifiable Fraction of *T. pyriformis* W lipids in Triparanol-Inhibited and Non-Inhibited Cultures.

Fraction	Relative retention time*	Control	Triparanol-inhibited
		(% composition)	
Peak A, squalene	.61	6.4	76.7
" B, unidentified	.96	16.5	12.6
" C, "	1.08		
Cholestane	1.00		
Peak D, unidentified	2.83	15.5	
" E, "	3.3		8.2
" F, tetrahymanol	7.6	62.6	2.0

* Relative retention time = ratio of retention time of a compound to retention time of cholestane.

and without triparanol citrate (1 μg/ml culture). The centrifuged cells were saponified, extracted with hexane, the hexane extract dried and chromatographed on the QF_1 column (chromatogram not shown). The per cent composition of the non-saponifiable components was calculated by determining the area of each peak in the total sterol sample. The results are shown in Table I. Squalene increased from 6.4% in the control to 76.7% in the inhibited culture while tetrahymanol decreased from 62.6 to 2.0%. Unidentified peaks B and C decreased. Peak D in the control was not seen in the inhibited culture whereas a new peak E (RRT 3.3, possibly from triparanol) appeared in the inhibited culture. In this one experiment, the very low amount of tetrahymanol (2%) in the inhibited culture might be erroneous as there is a possibility that tetrahymanol was not completely extracted by hexane. Furthermore, the 62% tetrahymanol in the uninhibited culture is lower than that (95%) observed by Connor.¶ Other work on the unidentified peaks and on the optimum extraction procedures for tetrahymanol is being carried out. Proteose peptone, in which the culture of *T. pyriformis* was grown, did not contain any sterol corresponding to the tetrahymanol peak on GLC or to the R_f value of tetrahymanol on TLC.

Discussion. It is apparent that marked changes occurred in the non-saponifiable fraction of triparanol-inhibited cultures of *T. pyriformis*. In animals, triparanol blocks the synthesis of cholesterol at desmosterol, although Holmes(12) has observed some increase in squalene and polyprenols in tissues of rats treated with triparanol. He suggests triparanol interferes with cyclization of squalene and our work with *T. pyriformis* confirms this. *Tetrahymena* is more plant-like than animal in the synthesis of the triterpenol, tetrahymanol; and the triparanol blockage of this synthesis at squalene constitutes presumptive evidence that squalene might be converted directly to the pentacyclic compound, tetrahymanol. Whether there are intermediates in this conversion remains to be proven through identification of the unknown peaks described above. It would appear that cholesterol is not in this pathway since cholesterol has not been observed in our work.

While the sterol requirement of some species of *Tetrahymena* has been well established(13), the composition of the non-saponifiable components of *Tetrahymena* have not been completely studied. Aaronson and Baker (14) did not observe a positive Lieberman-Burchard test in the unsaponifiables of *T. pyriformis* Type IIa. Connor¶ has not found cholesterol, although McKee *et al*(10), Seaman(15), Albach(16), Culbertson and Hull (17) and Taketomi(18) mention that *Tetrahymena* contains cholesterol or unspecified steroids. Castrejon and Hamilton(19) in a brief report in which glass-paper chromatography was used, found 4 sterols: dehydrolanosterol; another, unseparable from methostenol; one with an R_f identical with cholesterol; and one like zymosterol, in low concentration. The latter 2 disappeared after 3 days and dehydrolanosterol increased through the 6th day. In the light of the work of Mallory *et al*(9) it is likely that one of these is tetrahymanol.

We have no explanation for the reversal of triparanol-induced growth inhibition of *Tetrahymena pyriformis* mating Type II, variety 1 by some sterols (such as Δ7 cholestenol, 7 dehydrocholesterol and cholesterol among others)(6) unless these sterols could be used in an alternate pathway in the squalene-tetrahymanol synthesis or unless the ciliate steroid material of those organisms is not identical with that from *T. pyriformis* W. The observation that squalene, mevalonic acid and lanosterol failed to reverse tripara-

nol inhibition with *T. pyriformis* mating Type II(6) while squalene effectively reversed this inhibition with *Ochromonas*(2), also needs an explanation. The fact that fatty acids reverse triparanol-inhibition of growth of *Tetrahymena*(1-5) implies that a more fundamental process is affected. Kidder and Dewey(20) have suggested unconjugated pteridines as cofactors in the cyclization of squalene and in the desaturation of long-chain fatty acids. It is entirely possible that the changes reported here and previously, describing alterations in fatty acid composition(5), are brought about through an effect of triparanol on enzyme systems involving pteridines or other cofactors which mediate the above processes.

Summary. In triparanol-inhibited cultures of *T. pyriformis* (strains W, H and S), squalene is greatly increased in amount and tetrahymanol is decreased. In contrast, squalene appears in low concentration in uninhibited cultures. It appears that squalene might be converted directly to the pentacyclic compound, tetrahymanol, and that the cyclization of squalene is blocked by triparanol. Cholesterol was not identified in control or inhibited cultures and appears neither to be a sterol of major importance in this organism, nor an intermediate in the synthesis of tetrahymanol. Methods of purification and identification of tetrahymanol are given.

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Studies in Valine Biosynthesis VII. An Enzymatic Clue in *Penicillium chrysogenum* and Other Organisms.* (30066)

ROBERT L. WIXOM AND GEORGE B. HOWELL

Department of Biochemistry, School of Medicine, University of Arkansas, Little Rock

Penicillium chrysogenum and many other higher fungi can grow on ammonium salts as the main nitrogen source(1) and thus must have the enzymatic capacity to synthesize all of their amino acids. However, the pathway of valine and isoleucine formation has

* This investigation was supported by Grant RG-6626 of Nat. Inst. Health, U.S.P.H.S. Present address: Depts. of Biochemistry and Pediatrics, School of Medicine, Univ. of Missouri, Columbia.