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BIOSYNTHESIS OF CHOLESTEROL IN THE YEAST MUTANT ERG6

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SUMMARY: A mutant (erg6) of Saccharomyces cerevisiae defective in S-adenosylmethionine: Δ^{24}-sterol-C-methyl transferase (EC2.1.1.41) which normally produces cholesta-5,7,24-trienol and cholesta-5,7,22,24-tetraenol as the major sterols (total 4,4-desmethyl sterol content-8.3fg/cell) was shown to synthesize trace levels of cholesterol (0.08fg/cell). The identity of cholesterol was established by co-chromatography in TLC, GLC and HPLC with an authentic sample, mass spectroscopy and after an incubation with [1-^{14}C]acetate by isotopic dilution and recrystallization of the radiochemically purified material to constant specific activity.

Cholesterol is the major sterol end product of animal systems (1). In other non-photosynthetic and photosynthetic organisms the level of cholesterol appears to be under ontogenetic control so that it may be present as a trace or dominant sterol component depending on the organisms' developmental stage (2-4). While primitive fungi demonstrably produce cholesterol (5-7), the more-advanced fungi eg., Ascomycetes and Basidiomycetes, typified by the yeast Saccharomyces cerevisiae are generally thought to lack the genes for cholesterol production (8-10). The tacit assumption that yeast selectively alkylates the Δ^{24}-bond and cannot reduce it, a key step in cholesterol biosynthesis, has lead to a series of experiments on the importance of the biosynthetic inclusion of

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the C-24 alkyl group into the sterol side chain and yeast growth support (11-14).

These studies (11-13) agree on one point-that is a vitamin level of a "regulatory/sparking/synergist" sterol is required to initiate cell proliferations. The amount of the vitamin level sterol (added at 100 to 500ng/ml) if fully accumulated by growth arrested cultures (i.e., 1 to 5 x 10^8cells/ml), would be equivalent to 1.0-5.0fg/cell. The investigator's ability to determine this trace level of cellular sterol can be limited by the analytical methods of quantitation. Obviously, with a low mass of cells such hormone levels of sterol could easily be missed. Even when trace levels of cholesterol (ca., 0.1fg/cell) have unambiguously been characterized in a wild-type strain of S. cerevisiae by GC/MS, the investigators felt the need to place quotation marks about the compound (15). Unfortunately, most investigators, including ourselves, attributed reports on the occurrence of cholesterol in Ascomycetes and Basidiomycetes to laboratory contamination, or to have come from the culture media. The results described here show that cholesterol is a natural endogenously formed sterol of the yeast mutant erg6.

Materials and Methods

Yeast strain-A slant of the erg6 mutant was kindly provided by Dr. Martin Bard of Purdue University at Indianapolis. This strain was an erg6 segregant of strain JR5 originally created by Dr. Leo Parks of North Carolina State University (16).

Culture Conditions - The mutant was cultured in 1L flasks with 300ml of a peptone-yeast extract-glucose (2%:1%:2%) medium at 24°C. The cultures were shaken continuously at 200rpm on a G10 Gyrotary shaker bath for approximately 48h.

Sterol Analysis - Cells were harvested centrifugally from a growth-arrested population of 1.2 x 10^8cells/ml. They were ground with sea sand and 5% aq. acetone. The resultant acetone extract was dried then resuspended in a 25ml solution of water, methanol and KOH (10:80:10; v/v/w) and refluxed at 65° for 1h. The solution was diluted with water and extracted several times with ether. The residue from evaporation of the ether was dried by azeotropic distillation with benzene under reduced pressure and is referred to as the neutral lipid fraction (NLF). The NLF was chromatographed on silica gel G plates (Analtech, 250) and developed with benzene-ether (9:1) or a

portion directly injected into the GLC (3% SE-30 operated at 245°C). Bands of silica gel corresponding in R_f to cholesterol, lanosterol and cholesteryl oleate were scraped from the plate and extracted several times with 20ml portions of ether and methanol in a fritted glass funnel. The residue from the ether was dissolved in methanol for reversed-phase high performance liquid chromatography (RPHPLC). A 10μl sample was injected into a C_{18} column (11cm x 4.7mm, Whatman) maintained at 40°and eluted with MeOH: water (96:4) at 1 ml/min. The effluent was monitored continuously between 200 and 400 nm with an HP multiple wavelength diode anog detector (17). Retention times in GLC and RPHPLC are relative to cholesterol. GC-MS was performed at an ion source temperature of 150° as described (18).

Radioactive Experiments - The substrate was sodium [1-^{14}C]acetate, 1 mCi/1.55mg, obtained from NEN. Radioactivity in the samples was counted in Ecoscint cocktail on a Beckman LS5801. The labelled substrate was delivered to the experimental vessels prior to inoculation with cells. The cells were worked-up as in the erg6 cells used to identify sterols but after acetone extraction the TLE was saponified rather than spread on TLC to separate free sterols from esterified sterols.

Results and Discussion

The erg6 mutant of S. cerevisiae has been examined earlier for its sterol content (16,19,21). The mutant was shown to produce a series of cholestenols but not cholesterol itself. In our initial examination of the sterol composition of erg6 we noted by GLC (Fig. 1) two peaks which possessed RRT_c consistent with the previously characterized cholesta-5,7,24-trienol (major peak at RRT_c 1.18) and cholesta-5,7,22,24-tetraenol (minor peak at RRT_c 1.27). The

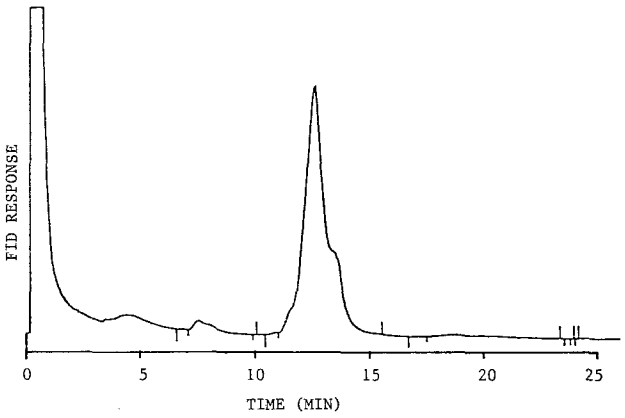

Fig. 1 GLC chromatogram of the total lipid extract of the yeast mutant erg6.

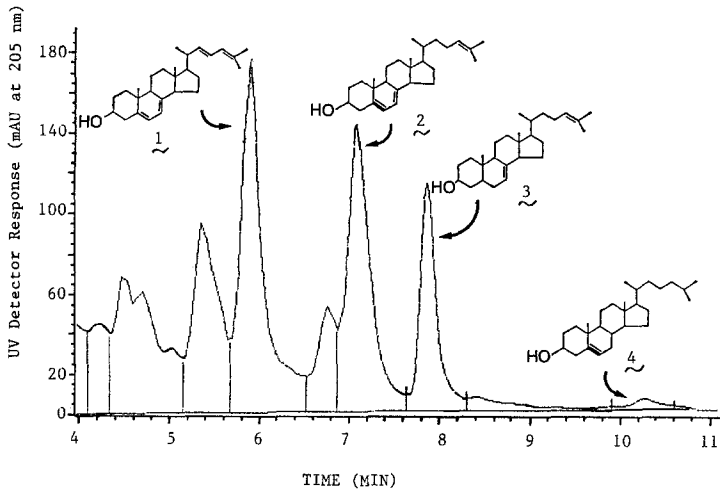

Fig. 2 RPHPLC chromatogram of the TLC purified 4,4-desmethyl sterols of erg6.

leading shoulder on the major peak possessed an RRT_c 0.89 which indicated a nonsteroidal compound. GC-MS of the two components eluting with RRT_c 1.18 and 1.27 possessed molecular ions of M/Z 382 and 380, respectively. These results were in good agreement with those reported in the literature (16,20). However, when the TLC purified 4,4-desmethyl sterol sample was injected into the HPLC we observed many additional peaks (Fig. 2). When the corresponding components were eluted from the column we determined the three major components 1,2 and 3 to be the previously identified cholesta-5,7,22,24-trienol, cholesta-5,7-24-trienol and cholesta-7,24-dienol respectively. We noted also a small component,4,eluted at a time characteristic for cholesterol and which showed a sharply vanishing end absorbtion in UV characteristic of Δ^5-sterols. When the fraction was examined by GC-MS, the main component of the GC record had a retention time exactly that of cholesterol and gave a mass spectrum identical with an authentic specimen (Fig. 3). In the earlier studies by Barton et al., the trace level of cholesterol could have easily been lost during the chromatographic separation and derivatization protocols. The

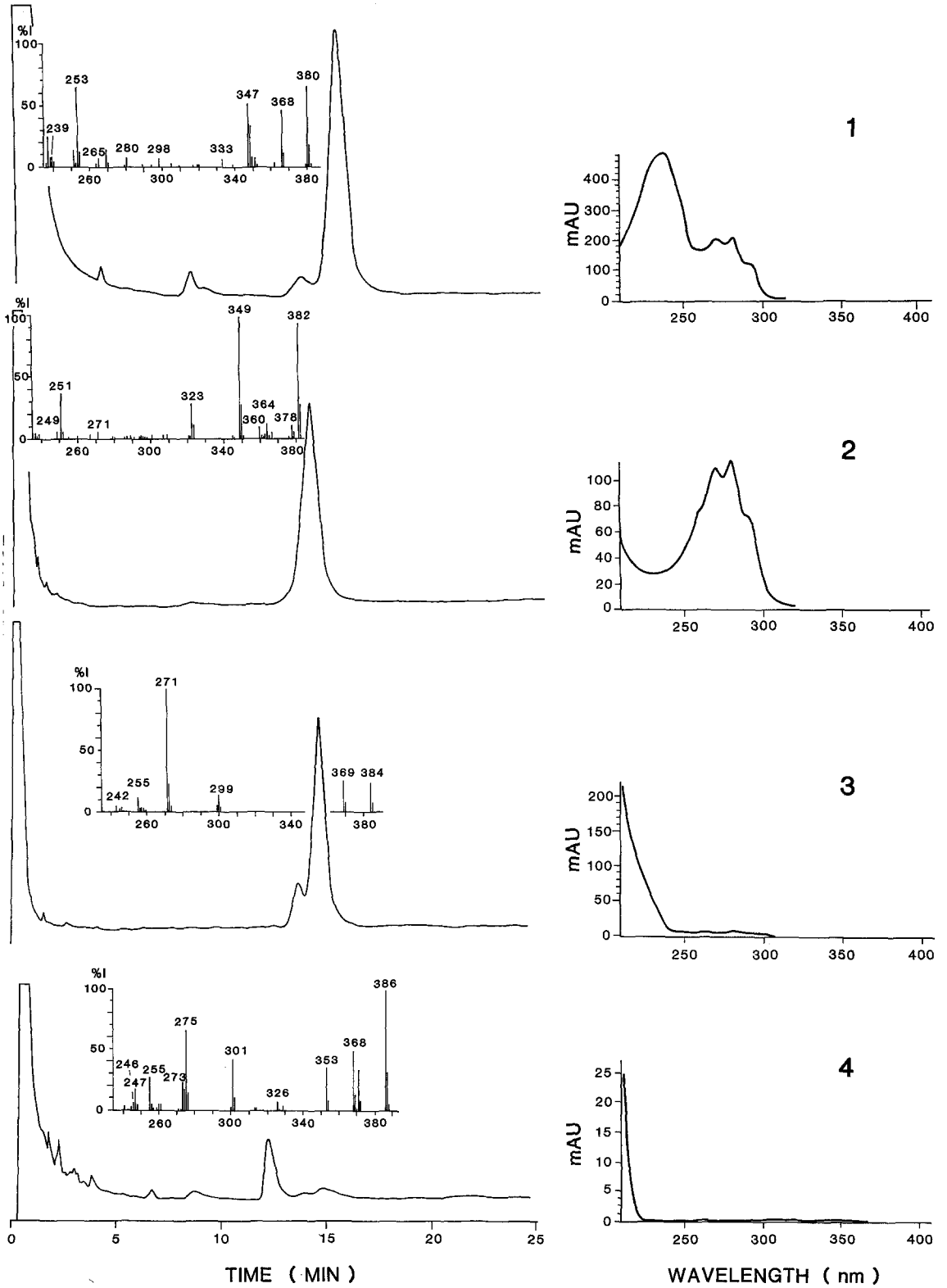

Fig. 3 GLC, MS (high mass region) and UV of HPLC purified sterols of the yeast mutant erg6. Spurious peaks in GLC were identified as fatty alcohols.

Table I. Incubation of erg6 yeast mutant with [1-^{14}C]acetate *

Material	Levels of Sterols and Radioactivity
Total Activity Added	2.07×10^{9}dpm
Total cells extracted	1.44×10^{11}
Culture volume	1.2 liter
Total mass of cells (wet)	19.8g
Average Mass/cell (wet)	1.38×10^{-10}g
Desmethyl Sterols Recovered	1.20mg
Cholesterol Recovered	0.012mg
Desmethyl Sterols/Cell (wet)	8.34fg
Desmethyl Sterol/100g cells (wet)	6.06mg
Cholesterol/100g cells (wet)	61μg
Total Radioactivity of NLF	1.23×10^{9}dpm
Total Radioactivity of Desmethyl Sterols	2.20×10^{7}dpm
Total Radioactivity of HPLC Purified Cholesterol	9.86×10^{4}dpm
Specific Activity of Total 4-Desmethyl Sterols	1.83×10^{7}dpm/mg
Specific Activity of Cholesterol	8.2×10^{6} dpm/mg

*one fg = 10^{-15}g.

cholesterol recovered from the cells was determined in different experiments conducted over a six month period to be at 0.08fg/cell ($\pm$0.04, n=5). This level was about two orders of magnitude lower than the combined cellular sterol content (Table 1).

At growth arrest the level of steryl ester was about 10 percent that of the free sterol. Cholesterol was also present as a minor component. Lanosterol (α_C 1.04, RRT_C 1.65, M^+426), the only detectable 4,4-dimethyl sterol, was present in trace amounts in both the free and esterified fractions. The bulk of the lipid material in the 4,4-dimethyl sterol fraction

rather than being steroidal was a series of long chain fatty alcohols (C-22 to C-30, confirmed by GC-MS).

In order to unambiguously demonstrate that the trace amount of cholesterol detected in HPLC was an endogenously biosynthesized sterol we proceeded with a [1-^{14}C]acetate incubation. In one experiment, a culture of 300 ml was supplemented with 50μCi of [1-^{14}C]acetate, an amount which labelled adequately the multiply unsaturated cholestenols. An aliquot of the TLC purified material was examined by HPLC-radiocounting of every one minute fraction. We detected mass but no radioactivity associated with cholesterol. Of course had we stopped our experiments here we would have erroneously concluded the cholesterol was not a natural erg6 sterol. When a large scale incubation was performed with 20 times the labeled substrate than the initial experiment and the mass associated with the peak corresponding to cholesterol carefully scaled-up after repeated injections into the HPLC, we obtained about 5μg of cholesterol (single peak in GLC). This material was diluted with 21.2mg of unlabelled cholesterol producing an initial specific activity of 3,113dpm/mg. No depression in the specific activity was noted after 3 recrystallizations each from hexane, methanol and acetone. A portion of this sample was reinjected into the HPLC (Fig. 4) and the radioactivity remained coincidental with the mass of cholesterol. In conclusion, the recent demonstration that wild-type S. cerevisiae possesses a Δ^{24}-reductase as shown by occurrence of 24,25-dihydrolanosterol (21,22) and 4,4,-dimethyl cholesta-8,14-dienol (22), coupled with this study proves that the genes for cholesterol synthesis are present in the erg6 mutant yeast. Its functional importance during the yeast life cycle remains to be determined.

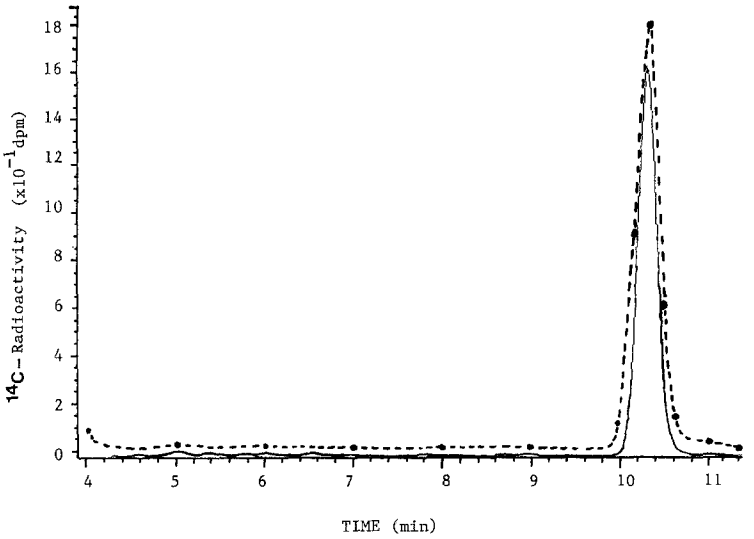

Fig. 4 Radiochromatogram of recrystallized cholesterol isolated from erg6 incubated with [1-^{14}C]acetate. The sample was dissolved in methanol and 90μg was loaded onto the column.

Acknowledgements

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