ANAEROBIC FORMATION OF ERGOSTEROL FROM A 5α-HYDROXYSTEROL BY CELL-FREE PREPARATIONS OF YEAST[1]

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The formation of cholesterol from Δ^7-cholestenol requires oxygen (Schroepfer and Frantz, 1961). 7-Dehydrocholesterol ($\Delta^{5,7}$-cholestadienol) is an intermediate in this transformation (Kandutsch and Russell, 1960), and the formation of 7-dehydrocholesterol from Δ^7-cholestenol is the oxygen-requiring step. Johnston and Bloch (1957) proposed that 7-dehydrocholesterol is formed from Δ^7-cholestenol via a 5α-, 6α-, or 6β-hydroxy-Δ^7-cholestenol intermediate. Neither of the 6-hydroxy epimers was dehydrated anaerobically by crude preparations of rat liver homogenate (Slaytor and Bloch, 1965). Preparation of 5α-hydroxy-Δ^7-cholestenol by these workers was not successful. However, others have prepared the corresponding 5α-hydroxy compound of the ergostane rather than the cholestane series. Ergosta-7, 22-diene-3β,5α-diol was prepared. Because ergosterol, the principal sterol of yeast, is the product of dehydration, the 5α-hydroxy ergostane derivative was incubated with cell-free preparations of yeast. The present report describes the anaerobic conversion of this diol into ergosterol by an enzyme from yeast that may be obtained in soluble form.

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Experimental methods.

Ergosterol was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. The crude sterol was crystallized from a mixture of methylene chloride and ethanol. Ergosterol acetate (pyridine and acetic anhydride) was irradiated in the presence of oxygen (Clayton et al., 1953). The resulting 5α,8α-peroxide of ergosterol was crystallized twice from chloroform and ethanol; m.p. 196-199°, $[\alpha]_D$-16°(c,1.0); reported: m.p. 199-206° and $[\alpha]_D$-19°(c,1.0); elemental analysis, $C_{30}H_{46}O_4$ requires: C, 76.55% and H, 9.85%; found: C, 76.52% and H, 9.79%.

The peroxide was reduced with lithium aluminum hydride, and the crude products were acetylated (Sivanandaiah and Nes, 1965). The 3β-acetoxyergosta-7,22-dien-5α-ol was isolated and crystallized from ethanol; m.p. 226-228°,$[\alpha]_D$+1.5°(c,0.7); reported: m.p. 228-233°,$[\alpha]_D$+2°(c,0.7) (Clayton et al., 1953). The acetate was saponified under reflux with 5% KOH in methanol. The product was isolated and crystallized from isopropanol (plates); m.p. 227-231°,$[\alpha]_D$+1° (c,0.5); reported m.p. 227-234°,$[\alpha]_D$+1° (c, 0.5); elemental analysis, $C_{28}H_{46}O_2$ requires: C, 81.50% and H, 11.23%; found: C, 81.54% and H, 11.14%.

Dry baker's yeast (Saccharomyces cerevisiae, Fleishmann) was purchased from Standard Brands, Inc., New York. Autolysis of the ground yeast was carried out exactly as described by Rilling and Bloch (1959). The remaining procedures were carried out at 4°. The suspension of autolysed yeast was centrifuged at 25,000 x g for 30 minutes. The supernatant fraction from centrifugation, called crude homogenate in this report, was decanted from the sedimented material and filtered through glass wool to remove floating lipid. The crude homogenate then was centrifuged at an average force of 105,000 x g for 2 hours. The supernatant

fraction was carefully decanted. Solid ammonium sulfate (enzyme grade, Mann Research Biochemicals) was slowly added to the supernatant fraction until a saturated solution was obtained. The precipitated protein was collected by centrifugation and dissolved in a small volume of water. The protein solution was dialyzed for 2 hours against water. Then the protein solution was dialyzed overnight against a large volume of 0.01 M phosphate buffer (pH 7.4). Similar precipitates of protein were obtained from solutions of ammonium sulfate that were between 0 and 25% and 25 and 100% of saturation.

The gelatinous microsomal pellets obtained from centrifugation at 105,000 x g were suspended in 0.05 M Tris-HCl buffer (pH 7.4).

Substrate (ergosta-7,22-diene-3β,5α-diol) was suspended in 0.05 M Tris-HCl buffer with Tween 80 as described previously (Gaylor, 1964). The final concentrations of the substrate and Tween 80 in the solution were 500 mμmoles and 10 mg per milliliter, respectively. One milliliter of the substrate solution was added to each incubation flask.

The preparations of the yeast enzyme were incubated with substrate in stoppered Erlenmeyer flasks. Anaerobic conditions were maintained by thoroughly equilibrating each buffer and the atmosphere with nitrogen. Glucose (40 mg) and 4 mg of glucose oxidase (Nutritional Biochemicals Corp.) were added to each flask to remove residual oxygen. The final volume of the incubation mixture was adjusted to 3.2 ml with 0.05 M Tris-HCl buffer. Incubations were carried out at 37° for 40 minutes. No added cofactors were required for activity.

Protein was determined by the method of Lowry et al., (1951). Formation of ergosterol was determined by extraction of the sterol from saponified mixtures following the incubation. The extracts were washed, dried over anhydrous sodium sulfate, and evaporated

to dryness. The residue was dissolved in 95% ethanol and the ultra-violet absorption spectrum was recorded with a Cary spectrophotometer. All conversions of the 5α-hydroxy compound into ergosterol were calculated from the net increase of ergosterol content of incubated samples compared to controls of either boiled enzyme or samples incubated without substrate. The absorption spectrum of the incubated samples was qualitatively identical to that of the controls. All conversions are reported as mμmoles of ergosterol formed.

Results and discussion.

The crude homogenate contained an enzyme that catalyzed the formation of ergosterol from the substrate (Table I). The yield of ergosterol was proportional to the amount of enzyme added.

TABLE I

Formation of ergosterol by various enzyme preparations from yeast.

Enzyme preparation	Protein added	Atm.	Ergosterol formed*
	(mg)		(mμmoles)
Crude homogenate	22	N_2	107
Crude homogenate	44	N_2	228
Supernatant fraction			
0 to 100% Am_2SO_4	18	N_2	123
0 to 100% Am_2SO_4	36	N_2	254
0 to 25% Am_2SO_4	34	N_2	216
25 to 100% Am_2SO_4	65	N_2	129
Microsomes	35	N_2	263
Microsomes	39	O_2	247
None	--	N_2	0

* average of duplicate samples from at least 3 incubations

An active preparation of soluble enzyme was obtained from the supernatant fraction following high speed centrifugation. The activity of this preparation was proportional to enzyme concentration. Further fractionation with ammonium sulfate yielded active enzyme in both major fractions investigated. Microsomes also contained active enzyme. Addition of oxygen did not affect the rate of formation of ergosterol. The ergosterol was not formed by non-enzymatic transformation of the substrate.

The time-course of ergosterol formation further verified the enzymatic nature of the transformation (Fig. 1). The reaction velocity was rapid during the first 10 minutes of incubation.

These results suggest that the 5α-hydroxysterol may be an intermediate in the formation of ergosterol. Other processes of formation of $\Delta^{5,7}$-sterols of the cholestane series were suggested recently (Paliokas and Schroepfer, 1967; Akhtar and Marsh, 1967). Further work on the purification of the soluble enzyme, the characterization of the reaction process, and the formation of ergosta-7,22-diene-3β,5α-diol is in progress.

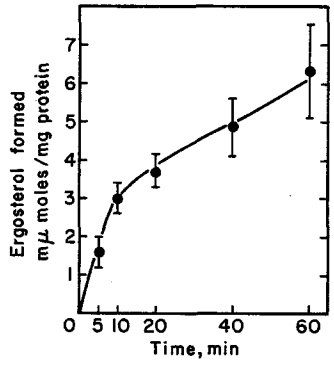

Figure 1. Time-course of formation of ergosterol by the 0 to 100% ammonium sulfate fraction.

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