ACCUMULATION OF A STEROL INTERMEDIATE DURING REACTION IN THE PRESENCE OF HOMOCYSTEINE WITH CELL-FREE EXTRACT OF YEAST*

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SUMMARY

D,L-Homocysteine, at the concentration of 10 mM, inhibited the methylation reaction of sterol side chain in cell-free extract of yeast, but did not inhibit ^{14}C-incorporation from [^{14}C]mevalonate into nonsaponifiable lipids. Under this condition, a radioactive C_{27}-sterol was accumulated. Examination by gas-chromatography on a DEGS column, partial hydrogenation, side chain cleavage, and by methylation with crude methyl transferase preparation, suggested the accumulated sterol to be 5α-cholesta-7,24-diene-3β-ol. The possible role of this sterol as a natural acceptor of the methyl group in ergosterol biosynthesis of yeast was discussed.

INTRODUCTION

The carbon atom in 28 position of ergosterol is derived from methyl group of S-adenosylmethionine in yeast but the natural methyl acceptor sterol has not exactly been characterized. Akhtar et al. (1) and Barton et al. (2) suggested that the methyl group in 28 position is introduced before the complete elimination of the three methyl groups attached to the sterol ring. In contrast, Katsuki and Bloch (3) demonstrated that ^{14}C-methylated sterol intermediates formed from [^{14}C]S-adenosylmethionine with crude cell-free extract of yeast had an ergostane skeleton, and they

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claimed that a C_{27}-sterol is the methyl acceptor. Moore and Gaylor (4) investigated the substrate specificity of S-adenosyl-L-methionine : Δ^{24}-sterol methyl transferase with the partially purified enzyme preparation. They found that C_{27}-sterols having at least a double bond between C-24 and C-25, especially zymosterol, served as a good acceptor and 4-methyl sterols were much poorer acceptors.

During the course of studies on the methylation of sterol side chain, we found that homocysteine inhibited the reaction in the cell-free extract. In this communication, we report the accumulation of a C_{27}-sterol, which is presumed to act as the methyl acceptor, in the reaction with the cell-free extract. A tentative structure of this sterol was proposed.

MATERIALS AND METHODS

[2 ^{14}C]D,L-Mevalonolactone (5.85 μCi/μmole) and [methyl ^{14}C]-S-adenosyl-L-methionine (58 μCi/μmole) were purchased from the Radiochemical Center, Amersham. D,L-Homocysteine and L-methionine were obtained from Nakarai Chemical Co., and NAD, NADP, ATP, GSH, glucose 1,6-diphosphate, and glucose 6-phosphate were from Sigma Chemical Co. Zymosterol is the gift of Professor C. Djerassi, Stanford University, and lathosterol (5α-cholesta-7-ene-3β-ol) is the gifts of Professor G. J. Schroepfer, Jr., Rice University and of Professor W. Klyne, Westfield College, from Steroid Reference Collection.

Methods of cultivation of Saccharomyces cerevisiae ATCC 12341, preparation of the cell-free extract, incubation conditions and of extraction of nonsaponifiable lipids were essentially the same as described previously (3). Radioactivities were measured in a Nuclear Chicago liquid scintillation spectrometer. The radioactive nonsaponifiable lipids from [^{14}C]mevalonate were separated on glass-fiber-paper impregnated with silica gel using benzene-dichloromethane (85:15) as described previously (3). Three major radioactive sterol fractions, S_A (Rf = 0.63), S_B (0.56) and, S_C (0.44), were eluted with benzene-methanol (1:1). The preparation of acetone powder of the cell-free extract and the enzyme reaction with sterol as substrate were done according to the methods of Moore and Gaylor (5). Radio-gas-chromatography was carried

out with a Shimadzu GC-5A equipped with a thermo-conductive detector, a Shimadzu FNC-1A furnace, and with a LSG-W22 flow detector. In this paper, a retention time was represented as a relative value to that of cholestane.

RESULTS

Inhibition of transmethylation reaction by homocysteine. From the expectation that the methyl-acceptor-sterol must be accumulated if the reaction is carried out in the presence of the specific inhibitor for the methylation reaction, various compounds were tested. Among them homocysteine fulfilled the requirement. Ethionine, norleucine, sarcosine, and betaine did not inhibit the methylation. At 10 mM, D,L-homocysteine inhibited ^{14}C-incorporation from [^{14}C]S-adenosylmethionine into nonsaponifiable lipids by 83% in the reaction with cell-free extract, but scarcely inhibited the ^{14}C-incorporation from [^{14}C]mevalonate. The distribution of radioactivities from [^{14}C]mevalonate in S_A, S_B, and S_C fractions on glass-fiber-paper chromatogram were 38.9, 21.3, and 24.2% in the incubation with homocysteine but 22.9, 19.0, and 42.6% in the incubation without homocysteine, respectively.

The autoradiogram of radioactive nonsaponifiable lipids derived from [^{14}C]S-adenosylmethionine on glass-fiber-paper gave only one radioactive spot with the same Rf value as S_C. The apparent inhibition by 10 mM D,L-homocysteine of the incorporation from mevalonate into S_C fraction was 43%, while the incorporation from S-adenosylmethionine was inhibited by 83%. This discrepancy suggested that not only methylated sterols but also other sterols might be present in this fraction. The radioactive sterols in S_A fraction were tentatively identified as lanosterol and 14-demethyllanosterol, and the sterol in S_B fraction as 4α-methylzymosterol. The assignment of these sterols will be reported elsewhere. No C_{27}-sterol was found in these two fractions.

Radio-gas-chromatography of S_C fraction. Radioactive S_C sterols formed from [^{14}C]mevalonate were, after elution from the glass-fiber-paper, analyzed by radio-gas-chromatography on a 1.5% OV-17 column. The chromatogram (Fig. 1A) shows that at least four radioactive sterols, S_{C1}, S_{C2}, S_{C3}, and S_{C4}, were present in S_C fraction. The incubation with 10 mM D,L-homocysteine (Fig. 1B)

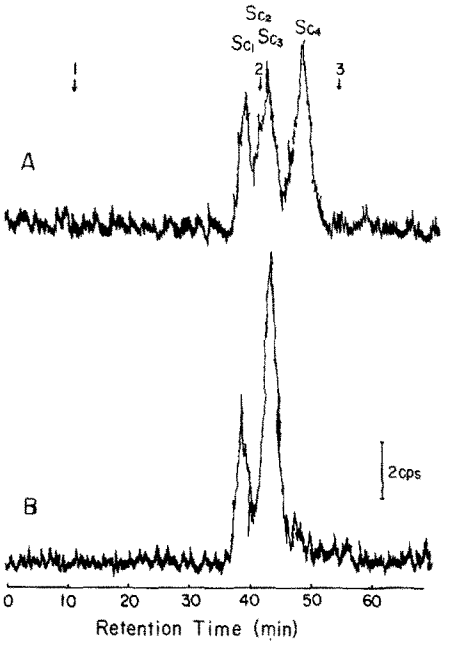


Fig. 1. Radio-gas-chromatograms of radioactive S_C fractions. [^{14}C]D,L-mevalonate (116 µmoles) was incubated with the following reaction mixture (final volume, 1.5 ml) for 6 hrs at 37° : 50 µmoles of GSH, 3 µmoles of $MgSO_4$, 4 µmoles of $MnSO_4$, 17 µmoles of ATP, 7.5 µmoles of NAD, 1.3 µmoles of NADP, 0.3 µmole of fructose 1,6-diphosphate, 8.4 µmoles of glucose 6-phosphate, 110 µmoles of potassium phosphate buffer (pH 7.4), 1.0 ml of cell-free extract of yeast (40 mg protein), and 10 µmoles of L-methionine (A) or 15 µmoles of D,L-homocysteine (B) as indicated. S_C fraction was prepared as described in the text. Both radioactive S_C fractions (96,000 dpm and 64,000 dpm in A and B, respectively) were, with standard sterols, injected into the gas-chromatograph. A glass column (1.5 m X 4 mm) packed with 1.5% OV-17 on siliconized shimalite W (60-80 mesh) was used at column temperature of 240° and at carrier nitrogen gas flow of 60 ml/min. The positions of the standard sterols in chromatographic pattern are shown by arrows: cholestane, arrow 1; ergosterol, arrow 2; and lanosterol, arrow 3.

gave rise to an increase of S_{C3} concomitant with a decrease of S_{C2} and S_{C4}, as compared with the control incubation (not shown). On the other hand, the incubation with 6.7 mM L-methionine brought about an increase of S_{C2} and S_{C4} concomitant with a decrease of S_{C3} (Fig. 1A). In order to determine the carbon skeletons of these sterols, they were hydrogenated with PtO_2 as catalyst in glacial acetic acid. By the treatment, the sterol preparation shown in Fig. 1B (actually composed of radioactive S_{C1} and S_{C3}) gave only radioactive cholestanol, whereas S_C preparation shown in Fig. 1A gave both of radioactive cholestanol and

ergostanol. When the enzyme reaction was carried out with [^{14}C]-S-adenosylmethionine as a radioactive substrate instead of [^{14}C]-mevalonate, two peaks with the same retention times as S_{C2} and S_{C4} were obtained. The hydrogenation of these two sterols gave only ergostanol. These results suggest that S_{C1} and S_{C3} are C_{27}-sterols, and S_{C2} and S_{C4} are C_{28}-sterols. The retention times of S_{C1} in gas-chromatography on three different columns ——1.5% OV-17, 1.5% SE-30, and 5% DEGS (as methyl ether)—— were coincident with those of the authentic sample of zymosterol.

Chemical structure of S_{C3}. For further characterization of S_{C3}, the number and position of double bonds in it were examined. The relative retention time of the methyl ether of S_{C3} on a 5% DEGS column was 5.70. This is in good agreement with the relative retention time of 5α-cholesta-7,24-diene-3β-ol (5.78) which was calculated from the observed values of several authentic sterol methyl ethers on this column according to the method of Clayton (6).

The existence of a nuclear double bond between C-7 and C-8 was confirmed by the partial hydrogenation with Raney nickel as catalyst. As shown in Fig. 2, the main radioactive peak produced from the mixture of S_{C1} and S_{C3} (1:4) was coincident with the mass peak of the added authentic sample of 5α-cholesta-7-ene-3β-ol.

The position of the double bond in the side chain of S_{C3} was determined by the oxidative cleavage with OsO_4 and $Pb(OAc)_4$. According to the methods described previously (3), the mixture of S_{C1} and S_{C3} (325,000 dpm) was oxidized with OsO_4 for three days at room temperature in ether and then treated with $Pb(OAc)_4$. The resulting mixture was distilled with 4 ml of acetone and the distillate was trapped by semicarbazide solution. Total radioactivity in acetone calculated from the specific radioactivity of recrystallized acetone semicarbazone (mp 187°) was 31,200 dpm. This value was about a half of the theoretical value (65,000 dpm). The fact that radioactive acetone was obtained from radioactive lanosterol in about the same degree of yield in a control experiment indicated the significance of the value of the yield. Thus it is evident that the double bond in the side chain of S_{C3} exists between C-24 and C-25. This was also supported by the methylation of S_{C3} being catalyzed by the extract of acetone powder prepared from yeast (5). After the incubation, radioactive peak of S_{C3} disappeared and a new radioactive peak appeared depending on S-adeno-

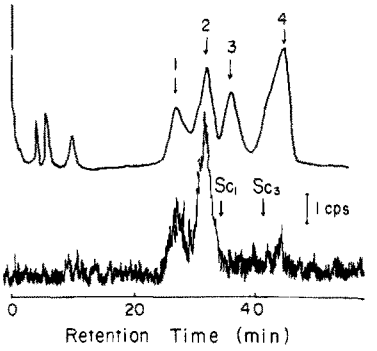


Fig. 2. Radio-gas-chromatogram of the partially hydrogenized S_C fraction. Radioactive S_C fraction (98,000 dpm) composed of S_{C1} and S_{C3} (the radioactivity ratio was 1:4), was incubated with 0.3 g of Raney nickel, 250 µg of lathosterol and 100 µg of cholestanol in 1 ml of ethanol under hydrogen gas at 30° for 60 min with vigorous shaking. The S_C fraction obtained was injected on a 1.5% OV-17 column at 245°. Mass peaks are cholestanol (arrow 1), lathosterol (arrow 2), ergostanol (arrow 3) and 5α-ergosta-7-ene-3β-ol (arrow 4). The original positions of S_{C1} and S_{C2} in the radio-gas-chromatogram are indicated by arrows for reference.

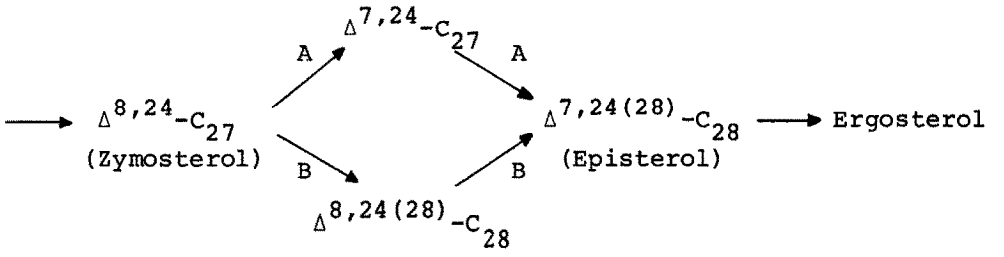
sylmethionine. The retention time of this methylated sterol was not coincident with that of the radioactive peak formed by the incubation of zymosterol with [^{14}C]S-adenosylmethionine in a similar manner.

DISCUSSION

In 1973, Fryberg et al. (7) proposed multiple pathways for ergosterol biosynthesis and they considered that the methylation occurs at the level of zymosterol or 4-methyl derivative of it and that episterol (5α-ergosta-7,24(28)-diene-3β-ol) is an important intermediate.

Parks et al. (8), very recently, reported that the methylation of sterol in yeast occurred mainly at the level of C_{27}-sterol and found the existence of a C_{27}-sterol, besides zymosterol which had been known as a sole C_{27}-sterol in yeast before. They proposed a tentative structure of 5α-cholesta-7,24-diene-3β-ol for the sterol. The present communication reported the accumulation of 5α-cholesta-7,24-diene-3β-ol by inhibition of the methylation reaction of the sterol side chain with homocysteine. Although no conclusive evidence has not been obtained suggesting that the sterol is the main acceptor for the methylation, it is attractive to speculate the

following pathway (route A) as an alternative or a main pathway of ergosterol biosynthesis:



In this regards, it is worth to note that a radioactive sterol with the same retention time as episterol is formed by the incubation for few minutes of intact yeast cells with [^{14}C]methionine (manuscripts in preparation).

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REFERENCES

1) Akhtar, M., Hunt, P. F., and Parvez, M. A. (1966) Chem. Commun. 565
2) Barton, D. H. R., Harrison, D. M., and Widdowson, D. A. (1968) Chem. Commun. 17
3) Katsuki, H., and Bloch, K. (1967) J. Biol. Chem. 242, 222
4) Moore, Jr., J. T., and Gaylor, J. L. (1970) J. Biol. Chem. 245, 4684
5) Moore, Jr., J. T., and Gaylor, J. L. (1969) J. Biol. Chem. 244, 6334
6) Clayton, R. B. (1962) Biochemistry 1, 357
7) Fryberg, M., Oehlschlager, A. C., and Unrau, A. M. (1973) J. Amer. Chem. Soc. 95, 5747
8) Parks, L. W., Anding, C., and Ourisson, G. (1974) Eur. J. Biochem. 43, 451