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Title: S-ADENOSYLMETHIONINE: Δ^{24}-STEROL METHYLTRANSFERASE AND THE REGULATION OF ERGOSTEROL BIOSYNTHESIS IN SACCHAROMYCES CEREVISIAE

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The inhibition of S-adenosylmethionine: Δ^{24}-sterol methyltransferase (EC 2.1.1.41) activity by endogenous cellular components has been studied in vitro. The principal inhibitors were Na^+ and K^+; Cs^+, NH_4^+ and Li^+ were also shown to inhibit the reaction. The inhibition by potassium cation has been studied, and we have found that energy dependent transport and accumulation of K^+ inhibits the sterol methyltransferase activity in intact respiring mitochondria.

Evidence is presented for three enzymatic activities capable of methylating sterols in cell-free extracts of yeast. These differ in their respective Michaelis constants for S-adenosylmethionine, pH optima, and affinity for zymosterol. An apparent cooperative-type relationship with S-adenosylmethionine has been proposed based on observed deviations from theoretical Michaelis-Menten kinetics. However, all three enzymatic activities were similarly inhibited by

monovalent cations, had the same subcellular location, and gave identical sterolic products.

The subcellular location of the sterol methyltransferase activity has been determined. All three activities reside in yeast mitochondrial and promitochondrial structures. This provides a direct relationship between ergosterol biosynthesis and respiration, since the mitochondria are the site of respiration in the cell. The sterol methyltransferase deviates from many other mitochondrial enzymes in that it is present at low levels during anaerobic growth and is not subject to catabolite repression by high levels of glucose.

S-adenosylmethionine: Δ^{24}-Sterol Methyltransferase and the Regulation of Ergosterol Biosynthesis in *Saccharomyces cerevisiae*

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INTRODUCTION

The synthesis and function of ergosterol in yeast has been the focal point of much research over the last 50 years. While the biosynthetic pathway leading from acetate to lanosterol (an ergosterol precursor) is well worked out and understood, relatively little is known of the conversion of lanosterol to ergosterol. Evidence now indicates a maze of pathways which will produce the end product, ergosterol. Likewise, little is known of the cellular function of ergosterol, except that it has a role in the maintenance of membrane structure.

In addition to this function, ergosterol has been demonstrated to be closely associated with the site of respiratory competency of yeast. Several mutants which have been isolated recently, and produce low levels of, or no ergosterol, either lack respiratory capabilities entirely or show a strong tendency towards petite induction. One exception is a class of nystatin resistant mutants which synthesize 8(9), 22-ergostadiene-3β-ol in place of ergosterol. The side chain of this compound is identical to that of ergosterol with only the phenanthrene ring being different. This could indicate that side chain maturation of ergosterol is one of the keys in determination of

the respiratory state of the cell. For this reason, the cellular controls over the terminal stages of ergosterol biosynthesis could also be important in deciding the cell's respiratory fate.

The enzyme catalyzing the sterol transmethylation reaction which supplies carbon-28 of ergosterol has been implicated indirectly as one point of regulation over the synthesis of ergosterol. In vitro experiments have shown that under certain conditions, ergosterol will inhibit this enzyme, suggesting a negative feed-back control. Furthermore, it has been reported that the suspected major substrate for this reaction, zymosterol, accumulates in yeast, whereas its methylated derivatives are further metabolized to ergosterol. Since this is a unique step in the overall synthesis of ergosterol, it is possible that this reaction could also be important in determining the respiratory fate of the cell. We have made an attempt to define more carefully the controls exerted over this important enzyme, in order to clarify its role in ergosterol biosynthesis.

LITERATURE REVIEW

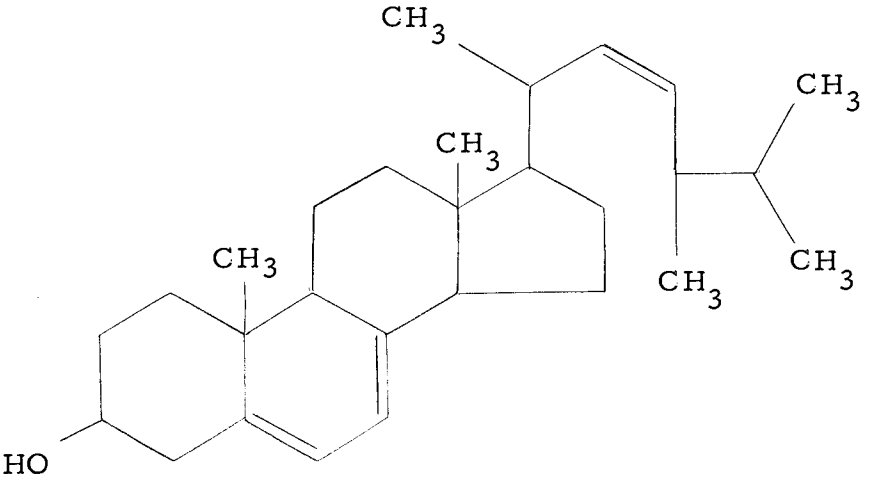
Ergosterol was first isolated by Charles Tanret in 1889 (78) from ergot of rye. He correctly determined values for it's melting point and specific rotation which have since been verified. Gerard (28) was the first to isolate the compound from yeast in an impure form. Smedley-MacLean and Thomas (76) isolated pure ergosterol from yeast of the genus Saccharomyces. They were able to isolate relatively large quantities of the compound and to this day yeast has remained the major source of ergosterol.

Ergosterol is the major sterolic component of fungi and yeast, having been isolated by Dulaney and his associates (25) from over 550 separate strains of yeast comprising 69 species in 20 genera. Ergosterol was found to average from 2-5% of the total dry weight of aerobically grown Saccharomyces cerevisiae. The carbon source was shown by Massengale (53) to be the principal factor in determining the degree of ergosterol production in yeast. These workers also found no correlation of the cellular sterol content with either total fat or nitrogen levels in growing cultures.

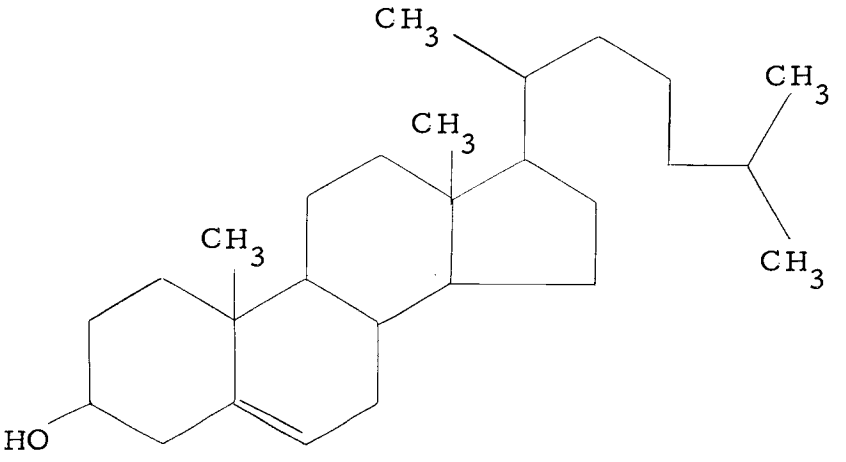
Rosenheim and Webster in 1927 (70) reported that provitamin D is an ergosterol-like sterol. This discovery initiated an intensive study of ergosterol and resulted in the elucidation of it's structure.

It was found to contain 28 carbons with unsaturation at positions five and seven in the B ring. Final analysis was finished in 1933 when Chuang (16) confirmed the structure of the nine carbon side chain of ergosterol. As Figure 1 shows, the structure of ergosterol is very similar to that of cholesterol. Other than the additional unsaturations at carbon 7-(8) in the B ring and at position 22-23 in the side chain, the only difference is the extra methyl group at carbon 24.

Establishment of the biosynthetic pathway for ergosterol in yeast has been accomplished through research in two directions. The first is essentially completed and concerns the synthesis of lanosterol from acetate. Maguigan and Walker (52) pioneered research on sterol synthesis in yeast in 1950. They showed that sterol synthesis occurred only during aerobic metabolism and that the carbon source directly affected the levels of sterol produced. More importantly, they noted that while acetate by itself gave reduced sterol production, when included with a sugar it resulted in as much as a 20% increase in sterol production. They concluded that acetate could satisfy the cell's need for carbon for use in sterol synthesis. Clayton and Bloch (17, 18) subsequently demonstrated that acetate could serve as the major carbon source for both ergosterol and cholesterol. Dauben and his co-workers (24) concluded that ergosterol was synthesized via squalene by studying the distribution of the



Ergosterol



Cholesterol

Figure 1. Structural relationship between ergosterol and cholesterol.

^{14}C label in ergosterol precursors biosynthesized from 1-^{14}C-acetate. Cornforth (20), by studying the individual enzymes, demonstrated that the ergosterol and cholesterol biosynthetic pathways through squalene cyclization were identical.

The second line of research has been directed at the conversion of lanosterol to ergosterol. This conversion entails demethylation at carbons 4 and 14, formation and positioning of unsaturation in the B ring and at carbon 22 in the side chain, and addition of carbon 28 to position 24. The origin of carbon 28 is unique with respect to the rest of the molecule. Hanahan and Wakil (31) analyzed the distribution of label in the side chain of ergosterol biosynthesized from carboxyl-^{14}C labeled acetate. They found none in the C-24 methyl group indicating a source other than acetate. Danielson and Bloch (23) demonstrated in 1957 that cells incubated in the presence of formate-^{14}C ended up with the label in ergosterol. They further showed that this label was specifically located in carbon 28 of the side chain. Alexander, Gold and Schwenk (8) incubated cell-free extracts of yeast with ^{14}C-labelled bicarbonate, formate, propionate, and methionine and found that methionine was by far the most efficient contributor to the 28th carbon of ergosterol. In 1958, Parks (61) showed that the actual methyl group donor in ergosterol biosynthesis is the activated form of methionine, S-adenosylmethionine.

Elucidation of the order in which the events leading to the conversion of lanosterol to ergosterol occur still remains something of an enigma. Except for Kodicek and Ashby's work in 1957 (45), most of the work on this problem has been done in the last 8-9 years. These two investigators let anaerobically grown cells undergo aerobic adaptation in the presence of 1-^{14}C-acetate while measuring the appearance of labelled sterols. They established with these experiments that the pathway went from squalene to lanosterol to zymosterol to ergosterol.

Although the origin of carbon 28 was shown to be from a transmethylation reaction via S-adenosylmethionine, it was still not known at what stage this reaction took place. Turner and Parks (85) demonstrated that this reaction was not the final step in ergosterol biosynthesis by isolating two methylated intermediates, one of which was partially converted to ergosterol upon being fed to whole cells. They reported their compound to be more polar than ergosterol as measured by column chromatography but gave no chemical structure for it. In 1967 Katsuki and Bloch (39) isolated two methylated intermediates, one of which appeared to be identical to that of Turner and Parks (85). They suggested that it's structure was that of a tetraene; 5, 7, 22, 24 (28)-ergostatetraene-3β-ol. Zymosterol was converted to the tetraene by intact anaerobically growing yeast cells. This led them to predict that methylation occurred after the demethylation

of lanosterol. Ponsinet and Ourisson (66) substantiated this claim by making a comprehensive study of methylated yeast sterols (4- 4 dimethyl, 14-methyl) related to lanosterol. They found no trace of any 24-methylene derivatives of this class of sterols. Recently, Parks et al. (60) have isolated a new methylated intermediate, 8(9), 22-ergostadiene-3β-ol from a nystatin resistant yeast mutant. When fed to wild-type yeast strains, this intermediate was readily converted to ergosterol.

Jauréguiberry (37) and his associates established that the methyl group of ergosterol retains only 2 of the 3 hydrogen atoms of the methyl group after transmethylation. Akhtar, Hunt and Parvez (4.5) studied the conversion of 24-^{3}H-lanosterol and 26,27-^{14}C-lanosterol to ergosterol. They showed both labels were incorporated at the same rate into ergosterol and that the ^{3}H atom had migrated from C-24 and was not located at C-23. From these experiments they predicted that the first step of the transmethylation procedes through a 24-methylene derivative and that a hydride shift from C-24 to C-25 is involved. They ruled out the possibility that a cyclopropane intermediate was involved in the alkylation of ergosterol. Russell and his co-workers (71) further substantiated this work by demonstrating that the $\Delta^{24(25)}$ bond is necessary in pea seeds for alkylation to proceed.

Moore and Gaylor (55, 56) have studied the transmethylation process in vitro using an acetone powder preparation. They tested lanosterol, desmosterol, zymosterol, 4α-methylzymosterol, and 4, 4-dimethylzymosterol for efficiency in accepting the methyl group. Zymosterol was found to be by far the most effective of the substrates tested, leading to the conclusion that it is the natural in vivo substrate for the reaction. However desmosterol, zymosterone, and cholesta -5, 7, 24-triene-3β-ol were methylated but at lesser rates.

The elucidation of the order in which unsaturation in the B ring and at position 22 is introduced has been much less successful. Topham and Gaylor demonstrated that ergosta 7, 22-diene-3β, 5α-diol could be converted to ergosterol in both crude cell-free preparations and with a purified 5α-hydroxysterol dehydrase enzyme (83). These experiments indicated that hydroxysterols could be intermediates in the formation of the $\Delta^{5,7}$-double bond system of ergosterol. They proposed a two step hydroxylation-dehydration mechanism requiring oxygen and pyridine nucleotide as in cholesterol biosynthesis. In 1972 (84) these same authors presented a reaction mechanism scheme entailing cyclopropane ring formation between the 3 and 5 carbons and using the above two-step process. This was in direct conflict with an earlier report by Akhtar and Parvez (6) predicting a cis elimination to form the double bond.

Much of the work done on the terminal stages of ergosterol biosynthesis has come from the laboratory of D. H. R. Barton (12, 13, 14). He has coupled organic syntheses of suspected ergosterol intermediates with yeast feeding experiments to predict a scheme for the later stages of ergosterol biosynthesis. In addition to Barton's work, Fryberg, Oehlschlager and Unrah (27) have studied the biosynthesis of ergosterol using similar feeding experiments. Based on relative incorporation frequencies they predicted that the major route from episterol to ergosterol involves the introduction of unsaturation at C_{22}, then at C_5, and finally reduction of the 24-methylene unsaturation. The concensus of this work indicates however, that there is no one pathway leading to ergosterol from lanosterol, but several different routes.

The regulation of ergosterol biosynthesis in yeast is an important aspect which has not been studied to any extent. In mammalian systems, cholesterol has been shown to feedback inhibit β-OH-β-methylglutaryl CoA reductase (75). In addition, at least two other sites have been shown to be similarly inhibited by Gould and Swyryd (30). Both the enzymes converting mevalonic acid to farnesyl pyrophosphate, and the one converting farnesyl pyrophosphate to squalene are likewise sensitive to feedback inhibition by cholesterol. However, in yeast, little is known of the regulatory mechanisms which function. Kawaguchi (48) has demonstrated that

the incorporation of radioactivity from 1-^{14}C acetate into the non-saponifiable lipids of yeast was repressed by the addition of ergosterol to the growth medium under anaerobic conditions. They found that four acidic lipids extracted from the yeast inhibited the cell-free incorporation of labeled acetate, but not mevalonate, into sterols. The structures of these lipids are unknown but it is possible that they resemble bile acids and are, in effect, ergosterol metabolites. They proposed that these lipids may play an important role in regulating ergosterol biosynthesis.

In addition, Moore and Gaylor (56) have shown that ergosterol has an unusual inhibitory action on the enzyme catalyzing transmethylation of zymosterol. At low zymosterol concentrations the rate of methylation was actually enhanced, but at higher substrate levels, ergosterol inhibited the reaction. This implies that ergosterol may negatively feedback inhibit this reaction in vivo.

The exact site of sterol synthesis within the cells is not entirely known. Kirtley and Rudney (42) observed that prolonged autolysis was necessary after freezing and thawing cells to obtain hydroxymethylglutaryl-CoA reductase in soluble form, which indicated a membrane attachment site. However, in 1969, Middleton and Apps (54) reported that two other enzymes involved early in the biosynthetic pathway were cytoplasmically located. Recently Schmizu and his co-workers (74) have shown that 3-hydroxy-3-methylglutaryl CoA reductase was located in yeast mitochondria. They further suggested

that the enzymes involved in the synthesis of mevalonate from acetyl-CoA, of farnesyl pyrophosphate from mevalonate and from farnesyl pyrophosphate to ergosterol, are located in the mitochondria, cytoplasm, and microsomes respectively.

The relationship between ergosterol production and aerobic metabolism has been well documented. Maguigan and Walker (52) showed that ergosterol synthesis is a function of oxygen utilization not related to or dependent upon cell growth. Simultaneous determination of ergosterol synthesis, cellular fermenting capacity, and oxygen consumption revealed that the formation of ergosterol coincided with the oxidative activity of the cell. Klein (43) observed that anaerobically growing yeast synthesize little, if any, sterol but that once aerated in the presence of a suitable carbon source, they produced large quantities of sterol. Tchenn and Bloch (79) demonstrated that under anerobic conditions squalene is accumulated intracellularly and that molecular oxygen is necessary for the removal of the extra methyl groups of lanosterol, the shifting of the double bonds in the nucleus, and as a source of the atom of oxygen in the 3-β-hydroxy group in sterol synthesis. Recently, Rogers and Stewart (69) found the sterol content of yeast to be linear with the medium's oxygen tension up to a level of 0.5μ M, while overall respiratory development increased linearly up to 3.5μ M oxygen. Thus, a small but

definite amount of sterols appear to be necessary for full respiratory development.

Although they evidently can't synthesize sterols under anaerobic conditions, yeast do require a preformed sterol (9) as well as an unsaturated fatty acid (10) during anaerobiosis. These authors found that sterols other than ergosterol would sustain growth (e.g., cholesterol), but to a lesser degree. The unsaturated fatty acid requirement was best satisfied by oleic acid.

Wild type strains of Saccharomyces cerevisiae possess both fermentative and respiratory mechanisms as means of deriving energy. Respiration occurs in the mitochondria. Loss of respiratory competency does not result in a loss of viability but rather in respiratory deficient organisms known as petites (57) and characterized by their small colony size when grown on glucose. A variety of enzymatic differences between wild-type and petite clones have been cataloged (26). These differences generally concern mitochondrial enzymes involved in respiration.

An intensive study has been made of the nature of mitochondria under anaerobic conditions and the development of respirationally competent mitochondria upon aeration of anaerobically grown cells. The existence of promitochondrial structures has been demonstrated (21, 58, 64) and recent experiments have shown them to be converted directly to respirationally competent mitochondria upon aeration (65).

Promitochondria from yeast cells cultured under anaerobic conditions and supplemented with ergosterol and oleic acid contain an extensively folded inner membrane system and well-developed cristae. Lipid deficient cells have mitochondria with inner and outer membranes, but no folded or internal membranes or cristae (38). These mitochondria have mitochondrial DNA, and the enzymes succinic dehydrogenase, malate dehydrogenase, and an oligomycin sensitive ATPase (88).

Much evidence has been accumulated which implicates an intimate relationship between ergosterol biosynthesis and respiration in yeast. Respiratory inhibitors such as dinitrophenol and cyanide have been shown by Adams and Parks (2) to stimulate sterol production in aerobically adapting cells. Elevated growth temperatures have been shown to induce a high percentage of petites in addition to an inhibition of sterol biosynthesis (77). However, the presence of ergosterol in the medium at these higher temperatures reduced by one-half the number of observed petites. Thompson and Parks (81) noted that ergosterol co-purified with cytochrome oxidase from yeast mitochondria. Although the sterol was unnecessary for _in vitro_ activity, a close _in vivo_ structural relationship is indicated between ergosterol and this enzyme of the respiratory chain.

The best evidence for an intimate association of ergosterol synthesis with respirational competence comes from the studies of

mutants. Alexander (7) and his co-workers found that the drug pentamethylenetrazol (metrazol) decreased the number and the cytological development of the mitochondria when included in the medium. The level of sterol in the cell was concomittantly reduced to that of yeast grown under microaerobic conditions. All mutants isolated thus far which are deficient in ergosterol production either lack respiration entirely or show an abnormally high frequency of petite induction with only one exception. This mutant produces $\Delta^{8,(9),22}$ ergostadiene-3β-ol in place of ergosterol (60). Recently several mutants have been isolated requiring methionine, sterol and an unsaturated fatty acid (11, 40). These mutants are also respirationally deficient. Evidence now indicates that these yeast may be lacking in the production of porphyrin compounds (11). Another mutant recently isolated by Gollub and his associates (29) requires only ergosterol and methionine, but has no fatty acid requirement. This mutant is also respiratory deficient.

A large amount of evidence now indicates that the lipid components of membranes modify the activities of many membrane functions (68). Manipulation of the membrane compositions by use of fatty acid auxotrophs has shown that the fatty acid composition directly affects the temperature dependent transition phase of cytochrome oxidase (3). Likewise, the effects of varying the cellular sterol concentration on the transition phase exhibited by mitochondrial

ATPase has been examined by Cobon and Haslam (19) and found to induce as much as a 17° change in the gel to liquid phase transition. A recent paper by Thompson and Parks (82) has shown that a change in the sterol composition of yeast also results in a change in the transition phase temperature for two mitochondrial enzymes. They utilized a nystatin resistant mutant which synthesizes 8(9), 22-ergostadiene-3βol in place of ergosterol for their experiments. They reported a 4-6° C change in the transition temperatures of both cytochrome oxidase and S-adenosylmethionine: Δ^{24}-sterol methyltransferase.

MATERIALS AND METHODS

Organism and Cultural Conditions

Saccharomyces cerevisiae, strain 3701-B, a haploid organism with a uracil requirement was used predominately throughout this study. The organism was maintained on agar slants containing 1% tryptone, 0.5% yeast extract, and 2% glucose. The yeast was grown aerobically in a similar broth in which 2% peptone replaced the tryptone. The glucose was replaced with 2% ethanol (v/v) when mitochondrial development was desired. The sugar was increased to 10% for complete repression of mitochondria. Cells were grown anerobically in 2-liter Erlenmeyer flasks containing 1800 mls. of a 1% yeast extract, 2% glucose, 0.1% NH_4Cl, 0.1 M phosphate buffer (pH6.5) medium, and equipped with Bunsen valves. A 10 ml. inoculum was added and the flasks sparged with N_2 through the cotton plugged arm of the Bunsen valve to remove residual oxygen. Anaerobically grown cells were adpated to aerobic conditions in Wickerham's complete medium (89) plus 5% glucose.

All cultures were incubated at 30°C.

Enzyme Preparation

The harvested cells were washed twice with 0.1 M Tris-Cl buffer, pH 7.5 and resuspended in the same buffer to 1g cells/ml.

All subsequent steps were carried out at 4°C. Twenty-five ml. of the cell suspension were added to a 75 ml. Duran flask and were broken in a Bronwill MSK tissue homogenizer using 40g. of 0.25 mm glass beads. The homogenate was centrifuged at 25,000 x g for 20 minutes and the pellet discarded. The supernatant was recentrifuged for 1 hour at 105,000 x g in a Spinco model L2-65 preparative ultracentrifuge. The floating lipid layer was removed with a Pasteur pipette. The supernatant was decanted and recentrifuged at 105,000 x g to remove any slowly settling particles. The clear supernatant from this centrifugation (designated S_{105}) was then stored at -20°C until used.

The sediment from the first 105,000 x g centrifugation containing the active sterol methyltransferase enzyme was resuspended to 15 mg protein/ml. in 0.1 M Tris-Cl buffer, pH7.5. Protein determination was done by the methods of Lowry et al. (50). This suspension was then stored at -20°C until needed. The enzyme is stable at this temperature for at least three weeks.

Isolation of Mitochondria

Cultures of 3701B were grown in an ethanol medium to mid-log phase and harvested by centrifugation. The cells were washed once in cold distilled water followed by a wash in 0.5 M sucrose, 0.1 M Tris-Cl, pH 6.5 buffer. The cells were then resuspended in the

same buffer to 1 g cells/ml. and broken as detailed above. Unbroken cells and cellular debris were removed by centrifugation at 2500 x g for 20 minutes. The mitochondrial-containing supernatant was then centrifuged at 25,000 x g for 20 minutes. The mitochondrial pellet from this centrifugation was washed twice in the sucrose buffer and recollected by centrifugation each time. The final pellet was resuspended to approximately 40 mg protein per ml. in the sucrose buffer and portions layered on linear 20-70% sucrose gradients. The sucrose gradients were also 20 mM in Tris-Cl, pH 6.5 and 2 mM in EDTA. The mitochondria were centrifuged for 2 1/2 hr. at 60,000 x g (middle of tube) in a Beckman SW 25.1 rotor. The gradients were fractionated by punching a hole in the bottom of the tubes and collecting 1 ml. aliquots. Density of the gradients was determined with a Zeiss hand sugar refractometer, model 0/30/0e.

Isolation of Promitochondria

Cultures of 3701B were grown in 10% glucose medium to late log phase and harvested by centrifugation. The cells were washed in cold distilled water, resuspended in 0.1 M Tris-Cl, pH 7.5, and broken as above. Unbroken cells and other cell fragments were removed by a 20 min. centrifugation at 25,000 x g. The supernatant was centrifuged at 105,000 x g for 1 hour and the resulting supernatant

discarded. The pellet was resuspended to a final concentration of 40 mg. protein/ml. in 0.1 M Tris-Cl buffer, pH 7.5 and homogenized in a Potter-Elvenhjem tissue homogenizer. 1.0 ml. of the homogenized preparation was layered on top of a linear 20-70% sucrose gradient and centrifuged for 16 hr. at 60,000 x g (middle of tube) in a Beckman SW 25.1 rotor. The sucrose gradient was also 0.02 M in Tris-Cl, pH 7.0 and 2 mM in EDTA. One ml. fractions were collected as with the mitochondria preparation.

Digitonin Fractionation of Mitochondria

The mitochondria were isolated as detailed above and suspended in 0.5 M sucrose, 0.1 M Tris-Cl buffer, pH 7.5 to a concentration of 15 mg. protein/ml. The digitonin method (36) for mitochondrial fractionation was used with slight modifications. Aliquots of ice-cold digitonin (10%, w/v) were added to the mitochondria to a ratio of 1.1 mg. digitonin per 10 mg. mitochondrial protein. This suspension was incubated for 20 minutes at 4°C, and then centrifuged at 15,000 x g for 10 minutes. The supernatant was decanted and saved. The pellet was washed with 10-15 ml. of the 0.5 M sucrose buffer, recentrifuged and this supernatant added to the first. The combined supernatants were centrifuged at 105,000 x g for 1 hour. The pellet from this ultracentrifugation (outer membrane) was resuspended in 0.1 M Tris-Cl buffer, pH 7.5. The supernatant (inter-membrane soluble fraction)

was dialyzed overnight at 4°C against 100 volumes of 0.1 M Tris-Cl buffer, pH 7.5.

The pellet from the first 15,000 x g centrifugation was resuspended to 15 mg protein/ml in distilled water and sonciated with a Branson sonic disruptor for 60 sec (3 x 20 sec) at 4°C and a power output of 85 amps. Digitonin was again added to the same ratio (1.1/10) and the suspension incubated 20 min. at 4°C. This suspension was centrifuged at 15,000 x g for 10 min. The supernatant was then centrifuged at 105,000 x g for 1 hr. to give the inner membrane portion in the pellet and the matrix portion in the supernatant. The inner membrane fraction was resuspended in 0.1 M Tris-Cl buffer, pH 7.5 and the matrix fraction was dialyzed overnight under the same conditions employed for the inter-membrane fraction.

Enzyme Assays

S-adenosyl-L-methionine: Δ^{24}-sterol methyltransferase was assayed by measuring the ^{14}C-methyl group incorporation from S-adenosylmethionine into the non-saponifiable lipids fraction. The assay tubes (25 x 150 mm screw cap culture tubes) contained 3.0 ml. of 0.1 M Tris-Cl, pH 7.5, 10 μmoles $MgCl_2 \cdot 6H_2O$, 0.8 μmoles of zymosterol, and 1.0 ml. of enzyme (diluted to 15 mg protein/ml.). Protein was determined by the method of Lowry et al. (50). 60 μmoles of $KHCO_3$ were added to the reaction mixture when the

105,000 x g pellet was used as enzyme source. The reaction was initiated by the addition of 0.125 μCi of ^{14}C-methyl-S-adenosylmethionine diluted with carrier substrate. The amount of S-adenosylmethionine added varied from 2.5 nmoles (0.58 μM final concentration) to 400 nmoles (93 μM) depending upon the K_m level under observation. The reaction volume was kept constant by varying the amount of buffer. The tubes were incubated at 30°C with gentle shaking for 30 min. after which the reaction was stopped by the addition of 2 ml. of 60% KOH. The mixture was saponified for 1 hr. and the non-saponifiable lipids removed by two 10 ml. extractions with n-hexane (2). The hexane was placed directly into scintillation vials, evaporated to dryness under N_2, 10 ml of scintillation cocktail (0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene in toluene) added, and the radioactivity determined in a Packard Tri-Carb 3214 scintillation spectrometer. All counts were corrected to 100% by the channels ratio method (34). The incorporation of the ^{14}C-methyl group of S-adenosylmethionine by cell-free extracts into the non-saponifiable lipid fraction was linear for at least 45 min. Zymosterol was always suspended in absolute ethanol just prior to addition to the reaction mixture.

Cytochrome oxidase was assayed spectrophotometrically by following the oxidation of cytochrome C at 550 nm (81).

Malic dehydrogenase was assayed spectrophotometrically by measuring the oxidation of NADH at 340 nm (86).

Measurement of S-adenosylmethionine

Freshly prepared 0.4 x 3 cm Dowex 50W-X8 (50-100 mesh) columns charged with LiCl were employed to determine S-adenosylmethionine levels before and after incubation with the S_{105} fraction. 1.25 nmoles of ^{14}C-S-adenosylmethionine were added to 1.0 ml of the S_{105} and the mixture incubated for 30 min at 30°C. Portions (0.2 ml.) removed at zero time and after 30 min incubation were applied to the Dowex columns. The columns were washed with 24 ml. of distilled water after which the ^{14}C-S-adenosylmethionine was eluted with 15 ml. of 3 N NH_4OH. One ml. samples from the water wash and the NH_4OH eluate were counted separately in 10 ml. of Bray's solution (15) on a Packard Tri-Carb liquid scintillation counter. Essentially all of the ^{14}C-S-adenosylmethionine added to the column was recoverable in the NH_4OH fraction.

Atomic Absorption Studies on S_{105}

A Perkin-Elmer 306 atomic absorption spectrophotometer was used to detect the presence of cations in the S_{105}. The presence of a particular cation was taken as a positive difference between the

absorption value for the S_{105} and that obtained for Tris-Cl buffer.

pH Curves

Buffers were prepared ranging from pH 5.5 to pH 8.5 and increasing by increments of 0.5 pH unit. All buffers were 0.1 M. At pH's 5.5, 6.0, 6.5 and 7.0, phosphate buffers were used, while a Tris-Cl buffer was employed at higher pH's. Overlapping of the two buffers used at pH's 6.5 and 7.0 was employed to correct enzyme activities to those obtained in Tris-Cl buffer only. Enzyme assays were then performed using the different buffers as described.

Solubilization and Partial Purification of the Methyltransferase Enzyme

Mitochondria were isolated and prepared up to the sucrose density gradient centrifugation step as described above. Sodium-deoxycholate was added to a final concentration of 2% deoxycholate and a protein concentration of 25 mg/ml. The pH was adjusted to 8.4 and the mitochondria incubated at $4^{o}C$ for 20 min. This solution was then eluted through a Sephadex G-50 column using 0.1 M Tris-Cl, pH 7.5 as the solvent. The major protein peak was collected and subjected to centrifugation at 105,000 x g. The supernatant was then used to obtain the protein fraction precipitating between 25 and 50% of $(NH_4)_2SO_4$ saturation. Most of the enzyme activity resided in the

pellet from this salt fractionation step. A sulfhydryl reducing compound was found to be necessary for stability at this stage and 20 mM glutathione was routinely used. At this stage there was a complete dependence of the enzyme for exogenously supplied substrate.

Substrate Preparation

Zymosterol was isolated from Fleischmann's cake yeast. The yeast were steamed for 2 hr. in 0.1 N HCl to break down the cell wall. This mixture was then cooled and made alkaline with solid KOH pellets added to 10% (w/v). The yeast were then saponified overnight in 50% methanol and 0.5% pyrogallol. The saponificate was neutralized with HCl to pH 7.0 and the non-saponifiable lipids were extracted into n-hexane, evaporated on a rotary evaporator, and the ergosterol-maleic anhydride adduct formed according to Schwenk et al. (73). The remaining sterols were extracted into alkaline methanol (10% KOH) which was then extracted with n-hexane and evaporated to dryness. The sterols were resuspended in a known volume of absolute ethanol-acetone (1:1) and then precipitated by the addition of an equal volume of 0.5% digitonin in 50% ethanol. The sterols were allowed to precipitate overnight at 4°C, collected by centrifugation, and washed twice with acetone-ether (1:1). The digitonide was split with dimethylsulfoxide (1), the zymosterol extracted with n-hexane, and evaporated to dryness. The sterol was recrystallized twice, once

with acetone followed by recrystallization from methanol. Purity of the zymosterol was determined by the presence of a single spot on thin layer chromatography (R_f 0.32) (60) and a melting point of 107-110°C which closely approximates reported literature values (72, 55).

Zymosterol-oleate was prepared by the methods of Knapps and Nicholas (44). The product gave a single spot on silica gel thin-layer chromatography (cyclohexane-ethyl acetate; 85-15) with an R_f = 0.56. Upon saponification in alkaline methanol (5% KOH), the ester gave only one sterol spot which corresponded with a zymosterol standard run simultaneously.

Potassium Flux Studies in Respiring Mitochondria

Intact yeast mitochondria were isolated by sucrose density gradient centrifugation as outlined above. All buffers used were maintained at pH 6.5 to prevent any possible damage to the mitochondria which might occur at alkaline pHs (46). Mitochondria were added to a 0.5 M sorbitol buffer, pH 6.5 to give a final protein concentration of 3-5 mg/ml. The buffer also contained Tris-maleate (10 mM), EDTA (0.5 mM), dialyzed bovine serum albumin (2 mg/ml.), and Tris-acetate (3 mM). Tris-succinate (3 mM) was added as an energy source and KCl (1 or 5 mM) added as indicated. The buffer was incubated with shaking at 30°C and samples taken and the K^+

levels measured by the methods of Haslam, Spithill and Linnane (32). A Perkin-Elmer, model 306, atomic absorption spectrophotometer was used to monitor K^+ levels. Valinomycin (50 μg) and 2, 4-dinitrophenol (0.1 mM) additions were made at selected intervals during the incubation period. Intra-mitochondrial methyltransferase activity of the respiring mitochondria was measured by removing and incubating 2.0 ml. portions of the sorbitol buffer in the presence of ^{14}C-methyl-S-adenosylmethionine. The incorporation of the ^{14}C-labelled methyl group into sterols was then determined as detailed above.

Materials

S-adenosyl-L-(Me-^{14}C)methionine (spec. act. 52 Ci/mole) was purchased from International Chemical and Nuclear Company. Unlabeled S-adenosyl-l-methionine was obtained from Boehringer-Mannheim. Cytochrome c (Type III), valinomycin, 2, 4-dinitrophenol, chloramphenicol, Silica gel G, sodium deoxycholate, Sephadex G-50, oxalacetate and NADH were purchased from Sigma Chemical Company. Digitonin and cycloheximide were purchased from Calbiochem. Dimethylsulfoxide was a product of the J. T. Baker Chemical Company. All other chemicals were commercially available and of the highest obtainable purity.

RESULTS

Preliminary investigations dealing with the sterol methyltransferase system and utilizing a crude cell-free extract as an enzyme source, had shown that portions of the 105,000 x g supernatant (S_{105}) added back to the assay system produced a marked reduction in methyltransferase activity. When the assay buffer was completely replaced by S_{105}, the transmethylation reaction was inhibited by 80-90%. Figure 2 shows the non-competitive inhibition resulting from substitution of 1.0 and 2.0 ml. of S_{105} for the buffer used in our assay system. The degree of inhibition by a given amount (generally 1.0 ml.) of S_{105} varied from preparation to preparation. Between 50 and 75% inhibition was generally produced by a 1.0 ml. aliquot of the supernatant.

The possible presence of an S-adenosylmethionine cleaving enzyme (35) in the S_{105} was investigated by allowing incubation of the S-adenosylmethionine with the supernatant for 30 minutes. Samples taken at the beginning and end of the 30 minute incubation period showed virtually no change in the S-adenosylmethionine level, thus eliminating the possibility of a cleaving enzyme. The possibility of non-sterolic methyltransferase substrates present in the S_{105} (i.e., tRNA, RNA, DNA) combined with a non-specific methyltransferase in the enzyme preparation was also investigated. The presence of

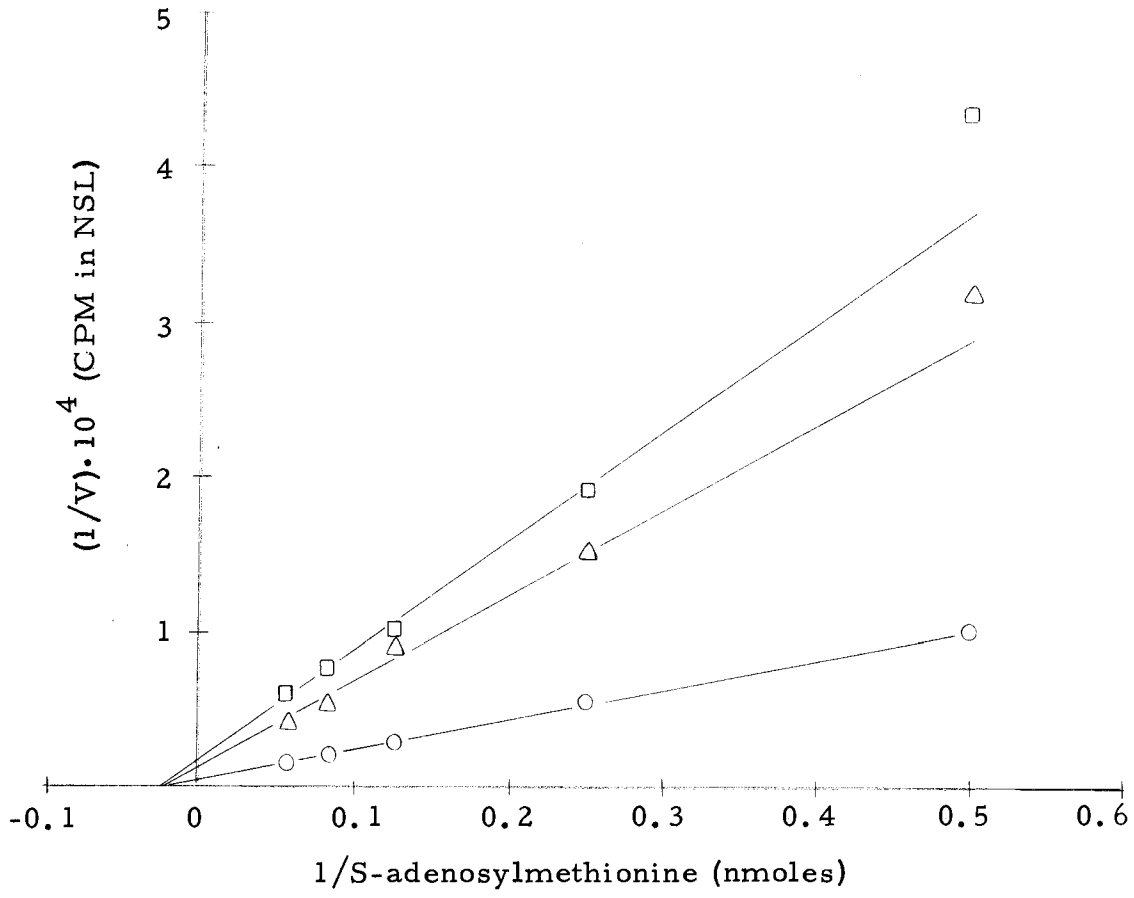


Figure 2. Non-competitive inhibition of the methyltransferase enzyme by endogenous inhibitor(s). The assay procedure is detailed in Materials and Methods. The transmethylation reactions were performed with varying S-adenosylmethionine (S-AM) concentrations. Reciprocal plots have been made of the transmethylation rates obtained with the enzyme alone (0-0), 1.0 ml of added S_{105} (Δ-Δ), and 2.0 ml of added S_{105} (□ - □).

such a methyltransferase would cause competitive inhibition. Figure 2 rules out this possibility since addition of the S_{105} results in typical non-competitive inhibition.

A series of experiments were then designed to investigate the nature of the inhibitory component present in the S_{105}. Table 1 shows the inhibitor to be completely removed by dialysis and to be heat stable to a temperature of 100°C for 1 minute. Prolonged heating of the S_{105} at 100°C still did not inactivate the inhibitor. To investigate further the nature of the inhibitory component, several 1.0 ml. volumes of the supernatant were ashed at 600°C for 2 hours. The ashing was performed in the same culture tubes used for the methyltransferase assay to prevent unnecessary loss of the ash. After resuspending the resultant ash in 1.0 ml. of Tris-Cl buffer, the enzyme assay reagents were added to the tube. Table 1 indicates that ashing removes only 30% of the inhibition. The majority of the inhibitory material thus appeared to be inorganic as evidenced by its stability to ashing.

Atomic absorption spectrometry was used to determine the nature of the cations present in the S_{105}. Known inhibitors of the methyltransferase enzyme such as copper and calcium (62) were not detected. However, sodium and potassium were found in the supernatant in large amounts. Potassium cation was found at a

concentration approaching 35 mM, while sodium cation was present at levels of 9-10 mM.

Table 1. Evidence for the Inorganic nature of Methyltransferase inhibitor present in S_{105}.
Various physical treatments of the S_{105} fraction are shown with the resulting inhibition obtained when 1.0 ml. of the treated S_{105} is added to the methyltransferase assay as described. Values shown are representative of three separate experiments.

Treatment of S_{105}	CPM in non-saponifiable lipids	Inhibition (%)
Control (minus S_{105})	23,100	-
Control (plus 1.0 ml. S_{105})	7,600	68
24-hr dialysis (against 200 vol. of 0.1 M Tris-Cl, 7.5)	23,600	0
Boiling (100°C for 1 min)	6,420	72
Ashing (600°C for 2 hr)	11,600	50

The possible inhibition of the sterol methyltransferase by these and other monovalent cations was then examined. Table 2 lists the cations tested showing the degree each inhibits the methyltransferase enzyme. In addition to the chloride salts used in Table 2, several other salts of each cation were tested. There was virtually no variance in the inhibition caused by these different salts. Sodium was by far the most effective inhibitor of the transmethylation reaction. A hyperbolic relationship between NaCl concentration and enzyme inhibition was observed with a maximum of 65% inhibition occurring

in the presence of 25 mM NaCl. The inhibition by different monovalent cations was found to be additive, and a solution of buffer made up to the sodium and potassium cation concentrations found in the S_{105} was found to produce a 45-50% inhibition of the methyltransferase enzyme.

Table 2. Inhibition of sterol methyltransferase by various monovalent cations.
Values reported are averages of two different experiments. Each salt is present in the enzyme assay at a concentration of 25 mM. In each case the percent inhibition is maximal for the particular cation.

Cation tested	Inhibition (%)
Na^+	65
K^+	35
NH_4^+	31
Cs^+	27
Li^+	32

Since 30% of the inhibition was removed by ashing, an analysis of the S_{105} for inhibitory organic compounds was made. The presence of ergosterol, a known methyltransferase inhibitor (5b), was detected in saponified portions of the supernatant. The ergosterol was found at levels approaching 30 μM. However, association of the ergosterol with small dialyzable cellular components was suspected since the S_{105} was refractory to hexane extraction without prior saponification. Glycine was also tested for inhibitory action (41) in our assay system with negative results.

While investigating the enzyme kinetics exhibited by the sterol methyltransferase enzyme as employed in our assay system, an unusual observation was made. Variation of the S-adenosylmethionine concentration produced atypical Michaelis-Menten kinetics as shown in Figure 3. The two plateau regions of the curve always appeared at the same relative substrate concentrations, and three different slopes were consistently observed between these plateaus. The insert depicts the velocity obtained at very low substrate concentrations and clearly shows a plateau between 1 μM and 1.5 μM S-adenosylmethionine. The second plateau exists between substrate concentrations of 27 and 30 μM. The same pattern was observed when only endogenous sterolic substrates were employed.

Because of this unusual kinetic data, several experiments were designed to investigate this observation in greater detail. Figure 4 shows standard Lineweaver-Burk plots of the three different slopes observed on the Michaelis-Menten curve (Figure 3). The average (3 experiments) apparent Michaelis constants (K_m) for S-adenosylmethionine are 4 μM, 32 μM, and 110 μM respectively. Figure 4 also shows all three phases to be inhibited by sodium cation in a noncompetitive fashion. The degree of inhibition of the methyltransferase reaction by sodium was independent of the substrate concentration and maximal inhibition was 65-70%.

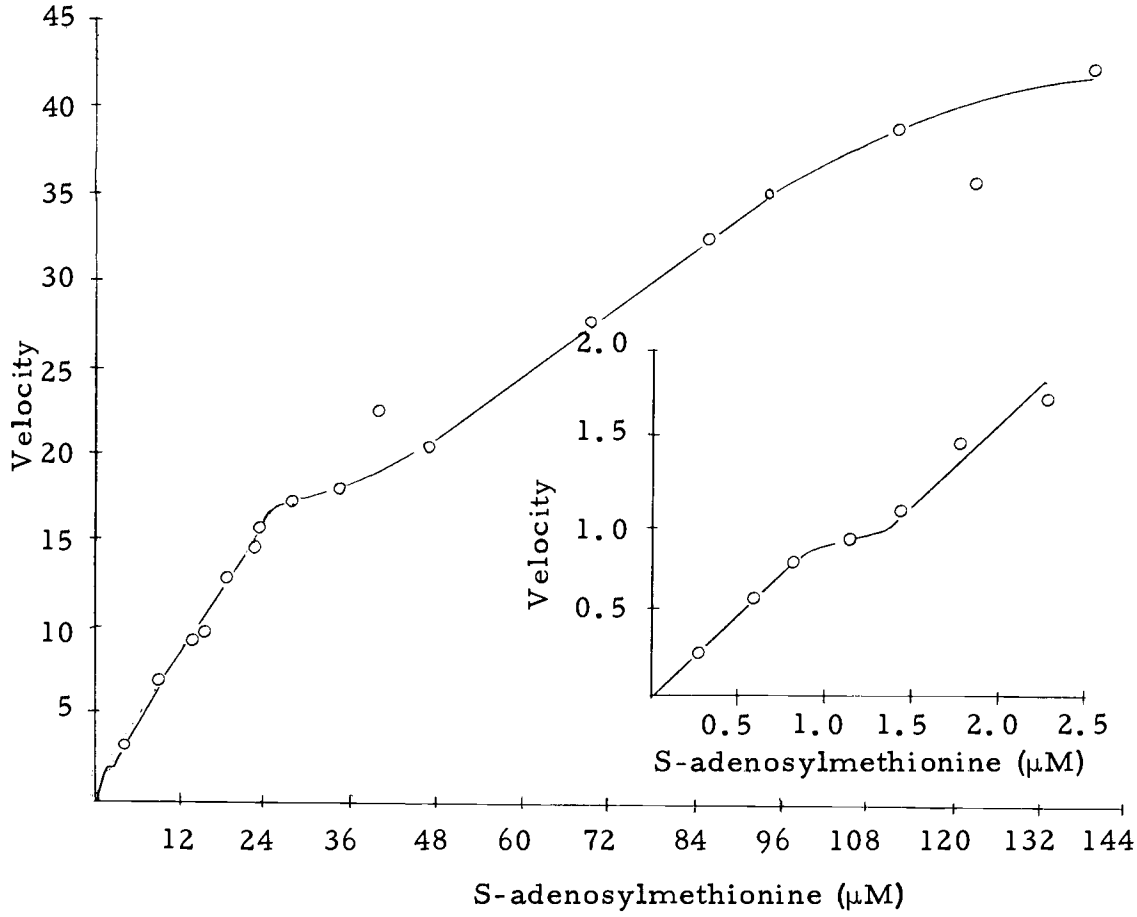
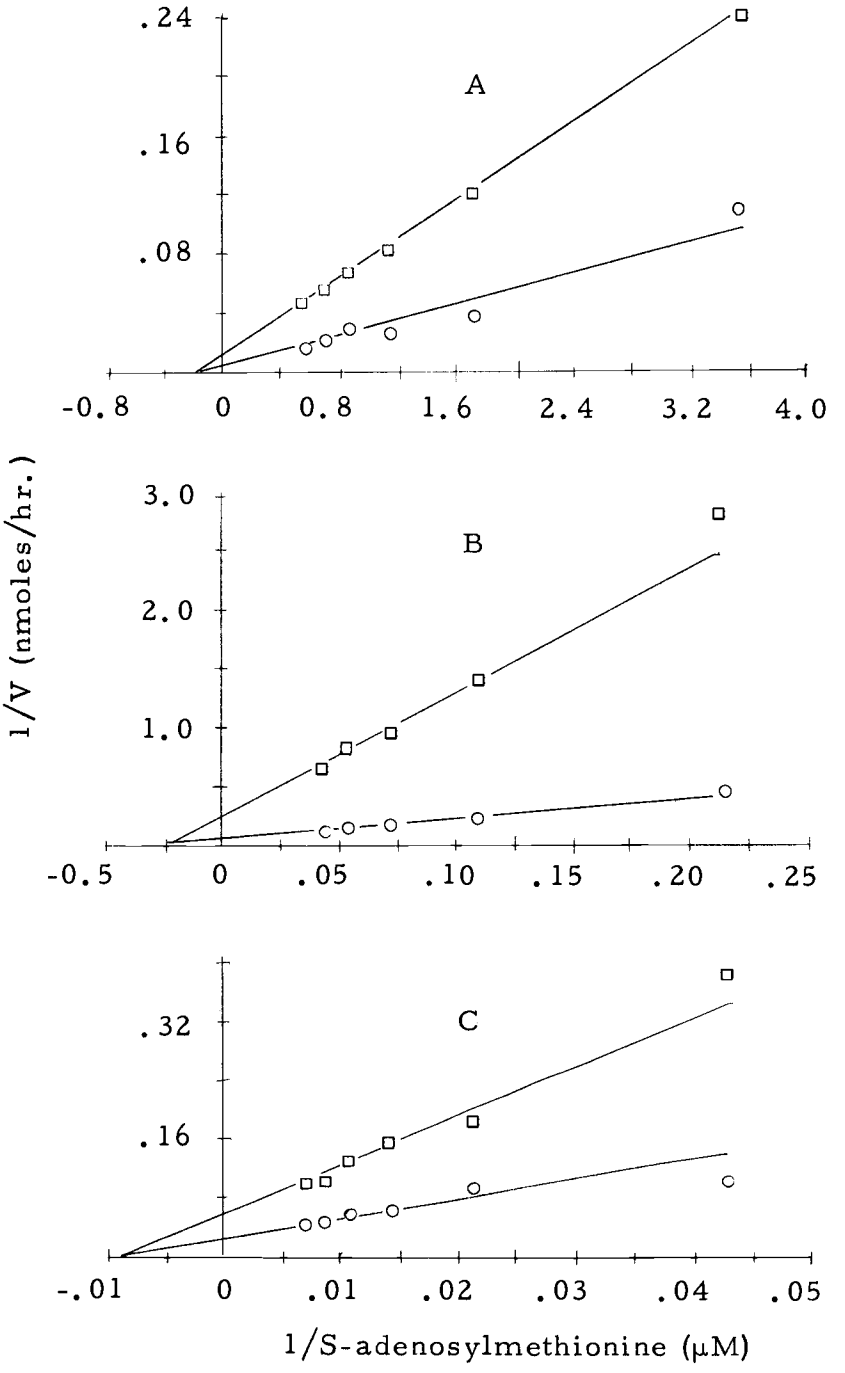


Figure 3. Michaelis-Menten plot of enzyme velocity against S-adenosylmethionine concentration. Velocity is expressed as nmoles product formed/hr/15 mg protein. The insert represents the lower substrate concentrations which cannot be detailed on the larger scale.

Figure 4. Lineweaver-Burk plots of the three slopes obtained from the S-adenosylmethionine substrate saturation curve (Figure 3). Enzyme assay procedure is detailed in Materials and Methods. S-adenosylmethionine concentration was varied from .29 μM to 1.5 μM in A; 1.5 μM to 30 μM in B; and 30 μM to 140 μM in C. Velocity is shown as CPM/hr/15 mg protein. The apparent K_m values obtained for this set of experiments were 3.5 μM for A, 38 μM for B, and 110 μM for C. (o-o) transmethylation rates with enzyme alone (□ - □) transmethylation rates in the presence of 25 mM NaCl.


.24
.16
.08
A
-0.8
0
0.8
1.6
2.4
3.2
4.0
3.0
2.0
1.0
B
-0.5
0
.05
.10
.15
.20
.25
1/V (nmoles/hr.)
.32
.16
C
-.01
0
.01
.02
.03
.04
.05
1/S-adenosylmethionine (μM)

The pH optima of the three different phases were investigated by assaying the methyltransferase enzyme activity in buffers ranging from pH 5.5 - 8.5 as detailed in the methods section. The results are represented in Figure 5. The highest enzymatic activity found in a given series of reactions was assigned a value of 100%. Percent activity as shown was thus calculated as a fraction of the "100% activity" assay. The optimal pH consistently varied from pH 7.0 to 7.7 with increasing S-adenosylmethionine concentrations. The pH of the reaction mixture remained constant throughout the incubation period.

Similar kinetic data found by other investigators with the carnitine palmityltransferase (35) of rat liver mitochondria lead to the subsequent characterization of two isozymes. Since our own data could also indicate the presence of more than one enzyme capable of methylating zymosterol, the exogenously supplied zymosterol concentration was varied to obtain K_m's for this substrate. At both the low (0.58 μM) and intermediate (10 μM) S-adenosylmethionine levels, an apparent K_m of 100 μM for zymosterol was obtained. However, at a substrate concentration of 93 μM, a K_m of 55 μM for zymosterol was found.

Subcellular fractionation studies were attempted next in order to determine the feasibility of possible methyltransferase isozymes having different cellular locations. Previous studies on the sterol

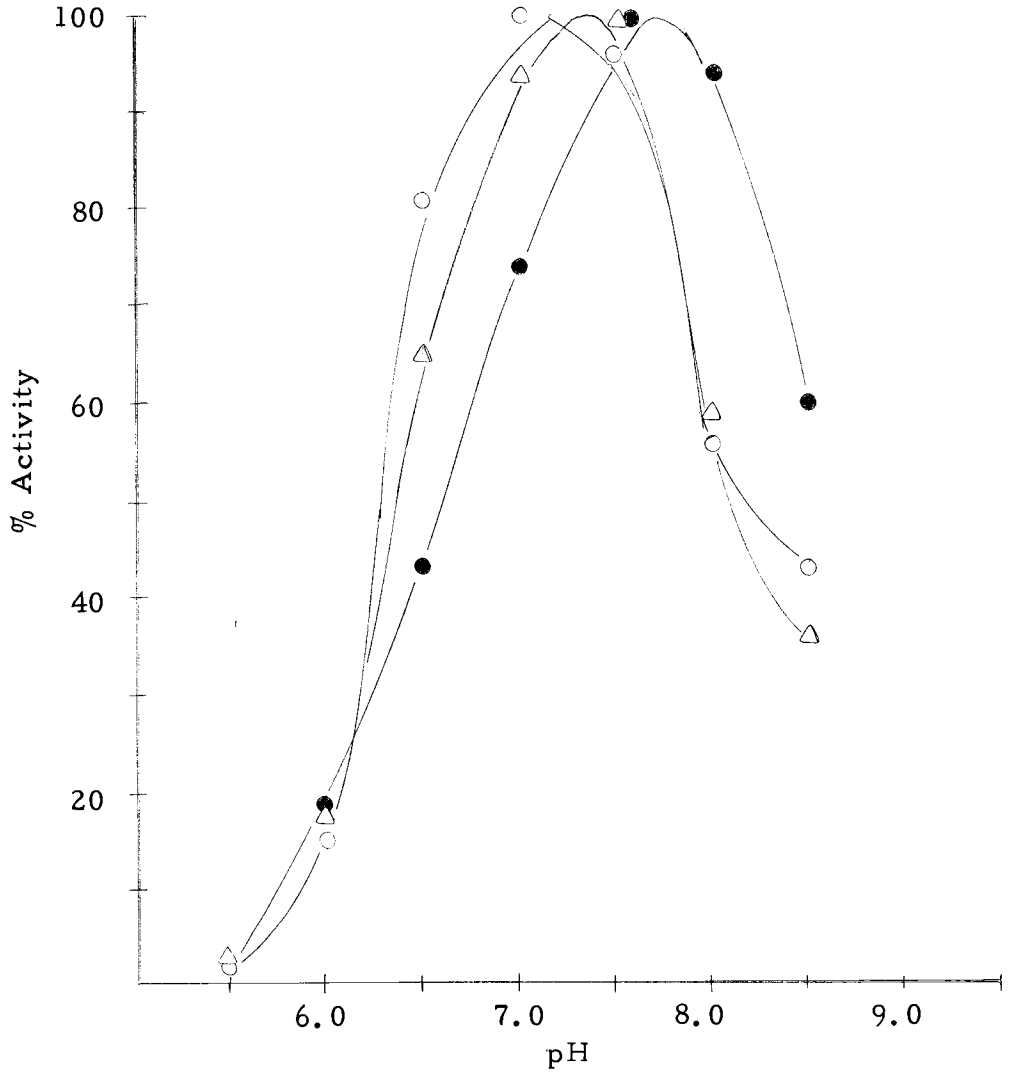


Figure 5. Optimum pH determinations for the three different methyltransferase activities. Both 0.1 M Tris-Cl buffer and 0.1 M phosphate buffer were used in these experiments. (o-o), 0.58 μM S-adenosylmethionine; (Δ-Δ), 10 μM S-adenosylmethionine; (●-●), 93 μM S-adenosylmethionine. pH optima were 7.1, 7.3, and 7.7 respectively.

methyltransferase in yeast were performed on cultures growing in glucose medium. We found that over 95% of the methyltransferase activity in these cultures was associated with the "microsomal" fraction (105,000 x g pellet from a 25,000 x g supernatant). This was true of all three of the methyltransferase activities described above. However, quite different results were obtained when the cells were cultured under conditions favorable for respiratory metabolism (ethanol medium). Under these conditions, the methyltransferase activity was found predominately in the pellet from the 25,000 x g centrifugation, or the mitochondrial fraction. Again all three of the enzymic activities were present in this fraction.

Sucrose density gradient centrifugation was employed to determine the purity and sedimentation of the mitochondrial fraction as isolated in the above paragraph. Cytochrome oxidase, a known mitochondrial enzyme (72), was used as a marker enzyme in these studies. Figure 6 shows that the A_{280}, cytochrome oxidase, and methyltransferase activities all banded at a density of 1.22 under the conditions used.

Since the methyltransferase enzyme activity was present in the mitochondrial fraction of ethanol grown cells, the logical location of the enzyme in glucose-repressed cells would be the promitochondria. Figure 7 shows a sucrose density gradient profile of the homogenized promitochondrial fraction from cells grown aerobically in 10% glucose

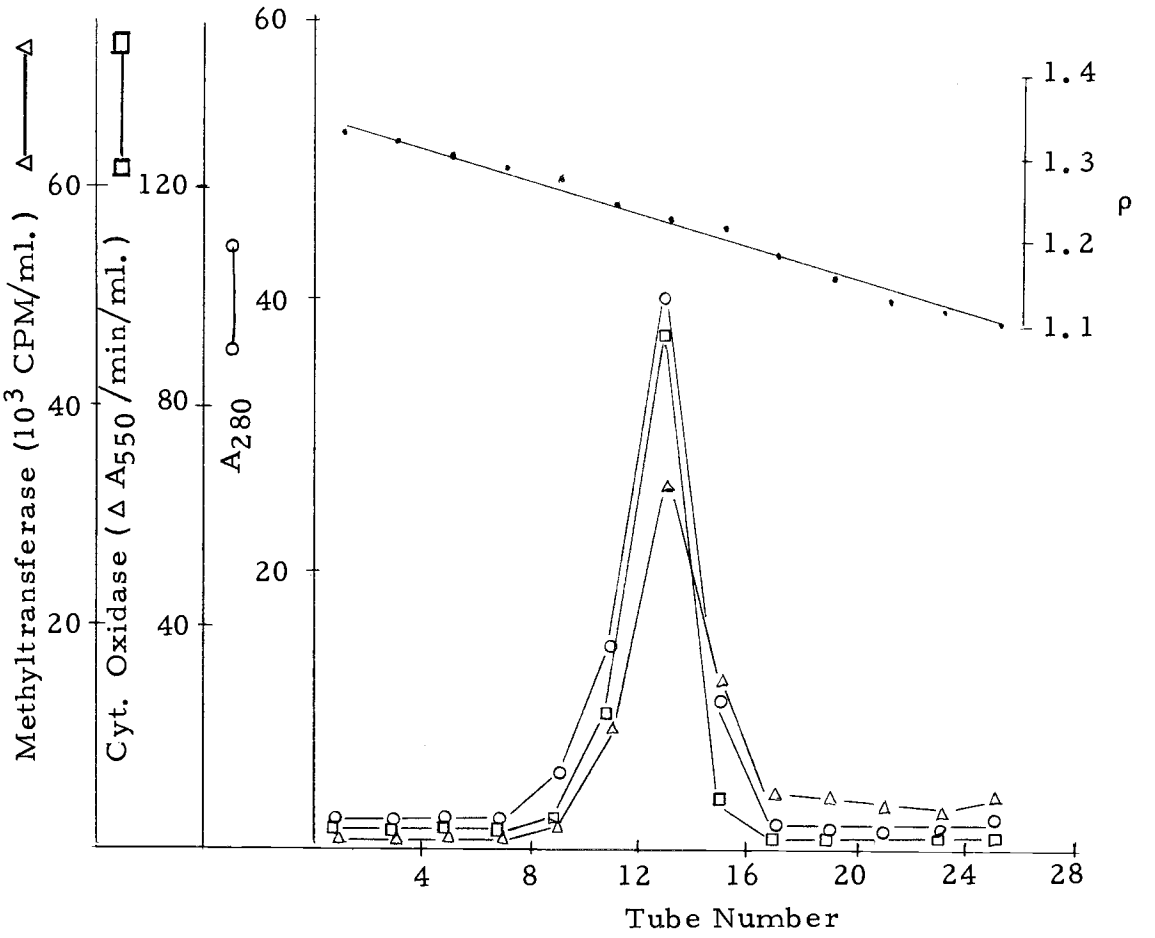


Figure 6. Sucrose gradient profile of isolated yeast mitochondria. Cells were grown under aerobic conditions with ethanol as principal carbon source. Mitochondria were isolated by differential centrifugation and resuspended to 40 mg protein per ml in 0.5 M sucrose, 0.1 M Tris-HCl buffer, pH 7.5. 1.0 ml of the mitochondria were layered on the linear 20-70% sucrose gradient.

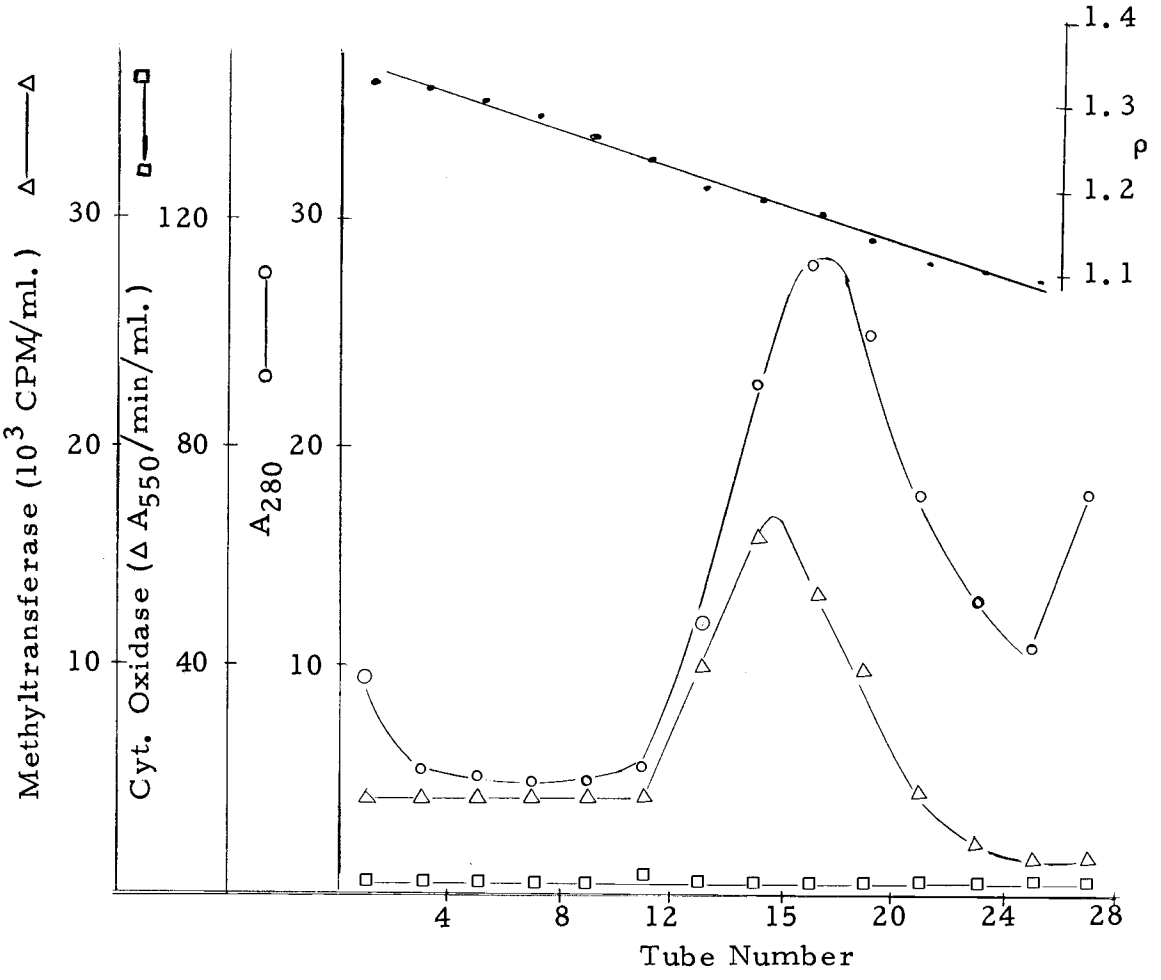


Figure 7. Sucrose gradient profile of isolated yeast promitochondria. Cells were grown aerobically in 10% glucose medium to repress mitochondria formation. Promitochondria were isolated by differential centrifugation, resuspended to 40 mg protein per ml in 0.1 M Tris-HCl buffer, pH 7.5, and homogenized extensively in an iced Potter-Elvehjem. 1.0 ml of the promitochondria were layered on top of the linear 20-70% sucrose gradient.

medium. As can be seen, the cytochrome oxidase activity is completely repressed, but methyltransferase activity is still present. The buoyant density value obtained for promitochondria was 1.18 which closely approximates published values (21). In conjunction with the promitochondrial location, we also looked for and found all three enzyme activities present during anaerobic growth.

Since all three of the apparent enzymatic activities were located in the mitochondria, the digitonin method of mitochondrial fractionation (36) was used to attempt the localization of the three possible enzymes in yeast mitochondria. Table 3 shows the results obtained with this method, using cytochrome oxidase and malic dehydrogenase as marker enzymes for the inner membrane and matrix fractions respectively. The sterol methyltransferase activity is obviously associated with both the inner membrane and matrix portions of the mitochondria. While Table 3 follows only one of the three sterol methyltransferase activities (K_m 110 μM), all three were similarly distributed within the mitochondria. A Michaelis-Menten plot exactly like Figure 3 was obtained when either the matrix or inner membrane fraction was used as the enzyme source, confirming the presence of the three enzyme activities.

Physical separation of the three enzymes also was attempted with no success. We used the solubliżed enzyme preparation for these studies as detailed in Materials and Methods. Table 4 details

Table 3. The distribution of sterol methyltransferase, cytochrome oxidase, and malic dehydrogenase enzyme activities in fractionated yeast mitochondria. The digitonin method of mitochondria fractionation is described in the Methods section. All enzyme assays were also performed as described in the Methods section.

	Cytochrome Oxidase		Malic Dehydrogenase		Sterol Methyltransferase	
Mitochondrial Fraction	ΔA_{550}/min/mg	% Total Activity	ΔA_{340}/min/mg	% Total Activity	CPM/hr/mg	% Total Activity
Outer membrane	1.47	7.9	2.7	2.5	265	11.7
Inter-membrane soluble fraction	0.42	0.7	20.0	5.7	105	1.4
Inner membrane	3.44	86.2	3.6	15.8	190	39.8
Matrix	0.91	5.2	76.0	76.0	1010	47.1

Table 4. Procedures used to partially purify S-adenosylmethionine: Δ^{24}-sterol methyltransferase from yeast mitochondria.
Purification values are based on intact mitochondria and not whole cells.

Stage of purification	Methyltransferase act.[1] CPM in non sap.-lipids	protein mg/ml	Spec act. CPM/mg protein
Intact mitochondria	7,060	8.0	880
G-50 eluent	5,640	6.5	870
Supernatant-105,000 x g.[2]	5,500	3.7	1490
Pellet-105,000 x g.	170	9.0	18
25-50% $(NH_4)_2SO_4$	26,300	5.2	5050

[1] The enzyme with a Michaelis constant of 4 μM S-adenosylmethionine is shown here but all three enzymes purify in the same manner.

[2] All assays were run in the presence of .02 M glutathione.

the purification scheme of the methyltransferase activity from yeast mitochondria. A 6-7 fold purification from whole mitochondria was attained by these steps. The enzyme maintained activity for only three days even in the presence of glutathione after these treatments. Several techniques including Sephadex G-200 gel filtration, DEAE ion exchange chromatography, and further salt fractionation were used in attempted separation of the three activities without success. However, we did find the three K_m's for S-adenosylmethionine to be present and precisely the same in this preparation which depended solely on exogenously supplied zymosterol.

Reports in the literature that a portion of the cells' zymosterol pool is esterified to predominately C-16 and C-18 unsaturated fatty acids (51, 59) led us to investigate the possibility of such a zymosterol ester being the preferred substrate of one or more of the three suspected enzymes. Accordingly, zymosterol-oleate was prepared by the methods of Knapps and Nicholas (44) as detailed in Materials and Methods. We used our solublized enzyme preparation which was free of any detectable endogenous substrate in these studies. When zymosterol-oleate was added to such a preparation and subsequently assayed at the three different S-adenosylmethionine concentrations, we found no significant incorporation of radioactivity into the non-saponifiable fraction. Table 5 summarizes these data. However,

when we tested the ester as a possible competitive type inhibitor of the methyltransferase reaction assayed with free zymosterol, we found to the contrary that it stimulated the reaction almost 2-fold. Figure 8 depicts a Lineweaver-Burk plot obtained by running the transmethylation assay with zymosterol alone, and with zymosterol plus its oleic acid ester. This occurred at all three substrate levels and no difference in the three K_m's was detected in the presence of the ester.

Table 5. Efficiency of Zymosterol-oleate as a substrate for the sterol methyltransferase reaction.
Sterol transmethylation assays were performed using either zymosterol or zymosterol-oleate as the substrate at each of the three different S-adenosylmethionine (S-AM) concentrations. The control tubes were run in the absence of any exogenously supplied substrate. All substrates were added at a concentration of 200 μM.

Substrate tested	CPM in non-saponifiable lipids		
	.29 μM S-AM	9.3 μM S-AM	93 μM S-AM
Zymosterol	5630	3660	3450
Zymosterol-oleate	400	240	290
Control	330	190	170

The sterolic products from the methyltransferase reaction remained the same at the different S-adenosylmethionine concentrations as measured by migration on thin-layer chromatography with Silica gel G (Benzene:ethyl acetate, 5:1). Only sterols which migrated

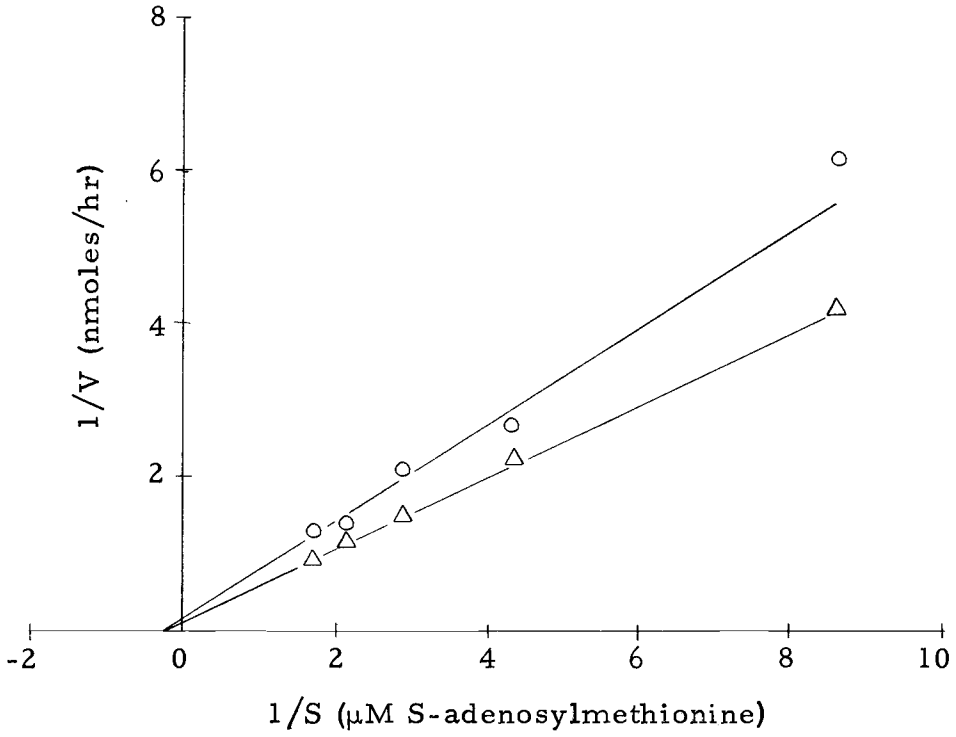


Figure 8. Stimulatory effect of zymosterol-oleate on sterol methyltransferase activity. Methyltransferase reactions were run using a partially purified enzyme preparation as detailed in Materials and Methods. Reciprocal plots of enzyme velocity as a function of S-adenosylmethionine concentration have been made. (o-o), 200 mM zymosterol; (Δ-Δ), 200 mM zymosterol plus 200 mM zymosterol-oleate.

at R_f's corresponding to fecosterol and ergosterol (60) (R_f = .33 and .13 respectively) incorporated the labeled methyl group in all three cases. It was noticed however, that the proportion of ^{14}C-ergosterol increased from 8% to almost 45% of the total non-saponifiable lipids with increasing S-adenosylmethionine concentrations.

Since the sterol methyltransferase activity in yeast was shown to reside in the mitochondrion, it is tempting to consider the sodium and potassium caused inhibition as a means of regulating sterol biosynthesis. To investigate this we studied the sterolic products from the methyltransferase reaction in both the presence and absence of potassium and sodium cations. Although both cations inhibited the overall incorporation of radioactivity into the non-saponifiable lipid fraction, neither caused any change in the ratio of labeled fecosterol to ergosterol when measured at a given S-adenosylmethionine level. This was interpreted to mean that only the transmethylation reaction is inhibited by the monovalent cations and not the further conversion of fecosterol to ergosterol.

Recently, several investigators have reported that a cyclododecadepsipeptide, valinomycin, induces potassium cation transport into isolated but respiring mitochondria (32, 46, 67). This technique provided us with a model system for actually measuring the effects of mitochondrial potassium levels on the sterol methyltransferase enzyme. Intact mitochondria were isolated from yeast and prepared

as described in the Method's section. These mitochondria were then incubated in 0.5 M sorbitol buffer with succinate added as an energy source. Valinomycin and 2,4-dinitrophenol were added sequentially and at time intervals samples were removed for both potassium measurement and assaying transmethylation activity. Figure 9 follows the variation in the internal mitochondrial potassium concentration which occurs throughout the experiment. Clearly valinomycin causes a rapid uptake of potassium into the mitochondria while the energy uncoupler, 2,4-dinitophenol, causes an efflux of accumulated potassium. More importantly, Figure 9 shows that there is an inverse relationship between transmethylation activity and the intramitochondrial potassium content.

Figure 10 shows that differential S-adenosylmethionine uptake into respiring mitochondria is not responsible for the changes seen in the sterol methyltransferase activity. Neither valinomycin nor 2,4-dinitrophenol present in the medium alter either the rate of uptake or the maximum level of S-adenosylmethionine taken into the mitochondria.

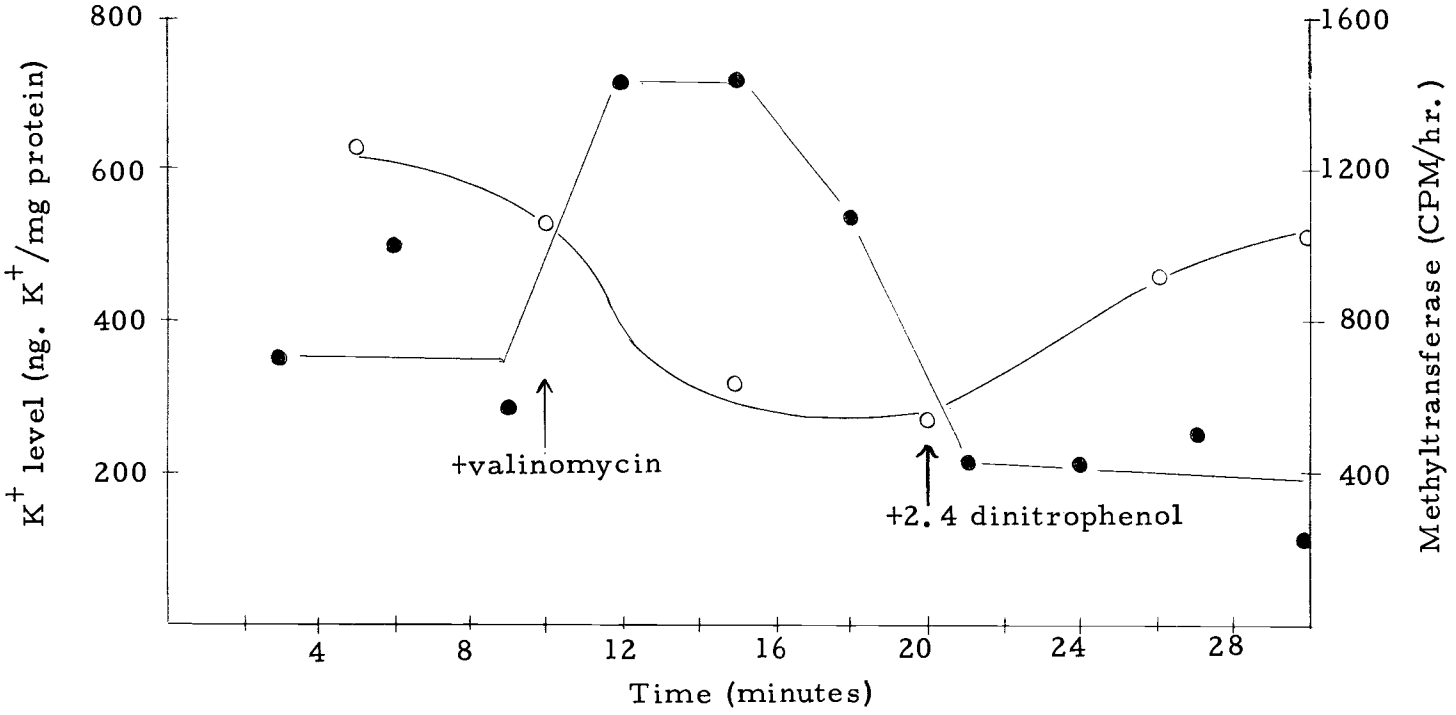


Figure 9. Induced potassium cation transport into the mitochondria and its effects on the methyltransferase activity. Mitochondria which were isolated and prepared as detailed in Materials and Methods were added to the 0.5 M sorbitol buffer (3.5 mg protein/ml) and incubated at 30°C. Samples were taken periodically and assayed for either potassium (●-●) or methyltransferase activity (○-○). Valinomycin (50 μg) was added at 10 minutes, and 2,4-dinitrophenol (0.1 mM) was added after 20 minutes incubation. The fluctuation of both the intramitochondrial potassium level and methyltransferase activity are plotted with time.

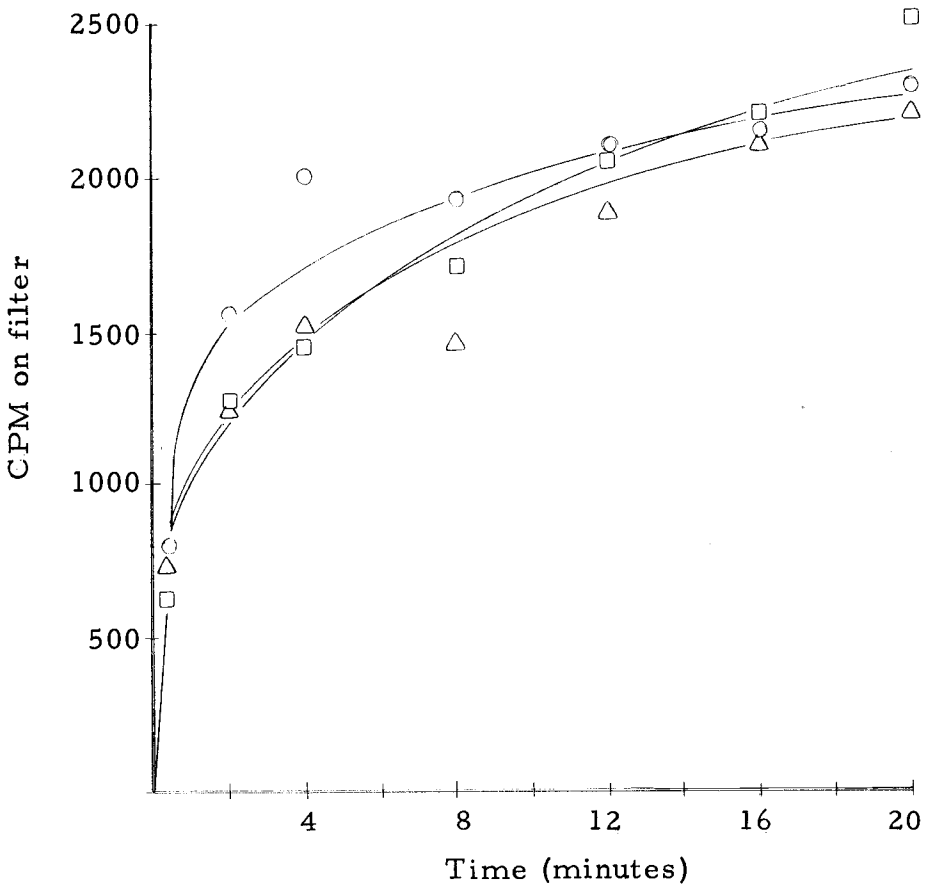


Figure 10. Uptake of ^{14}C-methyl-S-adenosylmethionine into respiring mitochondria. Mitochondria which were isolated and prepared as detailed in Materials and Methods were added to the 0.5 M sorbitol buffer (3-6 mg protein/ml) and incubated at 30°C. At time 0, 1.0 μCi of ^{14}C-methyl-S-adenosylmethionine (19 nmoles) was added and samples taken periodically. Each sample was filtered and washed with cold 0.5 M sorbitol buffer (10-15 seconds total time) after which each was assayed for radioactivity. (o-o), control; (□-□), plus 0.1 mM 2,4-dinitrophenol; (Δ-Δ), plus 50 μg valinomycin.

DISCUSSION

Several investigators (39, 55, 56, 85) have examined the sterol methyltransferase process in yeast with slightly conflicting results. While zymosterol has been shown to be the most efficiently used substrate (56), one group has reported that exogenously supplied zymosterol inhibited their cell-free methyltransferase enzyme preparation. Conflicting and varied co-factor requirements also have been reported (55, 85).

Addition of zymosterol to our cell-free enzyme preparation did not inhibit the methyltransferase activity as Katsuki and Bloch reported (85). To the contrary, added zymosterol greatly stimulated the reaction (6-fold). However, we found that zymosterol spontaneously breaks down as shown by the appearance of two distinct melting points. This resulted in a presumably unsuitable substrate which did inhibit the enzyme. Instead of the expected stimulation by the substrate, there was a considerable amount of inhibition imparted by the degraded zymosterol. The nature of the degradation is not known. For this reason, it was necessary to prepare zymosterol in absolute ethanol on the day it was to be used. It is relatively stable when stored at -15 ^{o}C as a powder.

The magnesium and carbonate requirements (85) were not absolute but both were necessary for full enzymatic activity. Like Moore

and Gaylor (55) we found a reducing agent necessary for maintenance of full activity once the enzyme was purified away from mitochondrial membranes. We routinely used glutathione although any of the reducing agents tested satisfied the requirement.

The non-competitive inhibition of the methyltransferase activity *in vitro* is due predominately to the presence of potassium (30-35 μM) and sodium (9-10 μM) in the S_{105} fraction. The inhibitors were completely dialyzable, heat stable, and partially stable to ashing at 600°C for 2 hours. The 25-30% loss of inhibition consistently observed after ashing indicates that some small molecular weight organic component of the S_{105} was also inhibiting the enzyme. Presumably, this organic inhibitor was ergosterol, a known methyltransferase inhibitor (56), which we found in the high-speed supernatant. It was found in concentrations approaching 30 μM. A "synthetic S_{105}" made up in Tris-Cl buffer containing both potassium and sodium, as well as ergosterol, all at their experimentally determined concentrations, inhibited the methyltransferase assay at precisely the same rate as the normal S_{105}.

The variation in inhibition by a given amount of different S_{105} preparations probably reflects the degree of cell breakage since the cations were presumable released during homogenization. Also, the resuspension to 1 g. cells/ml. prior to homogenization was dependent upon wet weight estimations and thus subject to some

variance. Thus the S_{105} was not standardized as far as cation or protein content from preparation to preparation.

The kinetic pattern obtained (Figure 3) by varying the S-adenosylmethionine concentration has several interesting ramifications. A similar, although only biphasic, Michaelis-Menten plot has been observed by other investigators for the enzyme carnitine palmityltransferase (36). These authors subsequently showed that their system was comprised of two separate enzymes catalyzing the same reaction, although there was relatively little difference between their respective Michaelis constants. The other general precedent for such a phenomenon involves the enzyme glutamate dehydrogenase (22). This enzyme exhibits a negative-type cooperative effect with its substrate wherein four separate Michaelis constants are observed.

It should be pointed out that similar saturation kinetics were observed when only endogenous sterol substrates were utilized. This would indicate that endogenous substrates were not being methylated preferentially at different velocities than the exogenously supplied zymosterol.

The kinetic data for the three enzyme activities are summarized in Table 6. It appears that there may be three different enzymes present in yeast capable of methylating zymosterol. The enzymes differ on the basis of their affinity for S-adenosylmethionine, pH optima, and apparent Michaelis constants for zymosterol. The

Table 6. Summary of kinetic data for S-adenosylmethionine: Δ^{24}-sterol methyltransferase activities.

Enzyme activities were arbitrarily designated 1, 2, and 3 for reference purposes. V_{total} was calculated by extrapolation to infinite substrate concentration from Lineweaver-Burk plots (Figure 4). Each individual V_{max} was then determined by subtracting the V_{max} of the preceding enzyme(s) (next lower K_m values) from the V_{total} value.

Methyltransferase Activity	pH optimum	K_m Zymosterol (μM)	K_m S-adenosyl. (μM)	V_{max} (nmoles prod/hr/15 mg)
1	7.1	100	4	5
2	7.3	100	32	26
3	7.7	55	110	49

S-adenosylmethionine concentrations used to determine zymosterol K_m values were those calculated to give a minimal contribution to the velocities of the other enzyme activities.

The lower zymosterol K_m for the third enzyme implies that it is more specific towards zymosterol as a substrate than the other enzymes. Both the pH optimum of 7.7 and the Michaelis constant for zymosterol (55 μM) of this enzyme agree well with previously reported values. Moore and Gaylor (55) found a pH optimum of 7.5 and a zymosterol K_m of 62.5 μM using similar substrate levels in an acetone powder preparation.

An enzyme which exhibits strict Michaelis-Menten kinetics would be expected to follow the following equation:

$$v = \frac{V_{max}(S)}{K_m + (S)}$$

By rearranging the above equation and extending it, the following equation may be derived for a system containing three enzymes and following Michaelis-Menten kinetics:

$$V_{total} = \frac{V_{max}(1)}{1 + \frac{K_m(1)}{S}} + \frac{V_{max}(2)}{1 + \frac{K_m(2)}{S}} + \frac{V_{max}(3)}{1 + \frac{K_m(3)}{S}}$$

The numbers 1, 2 and 3 refer to the values for the three respective enzymes as shown in Table 5. Figure 11 depicts a theoretical

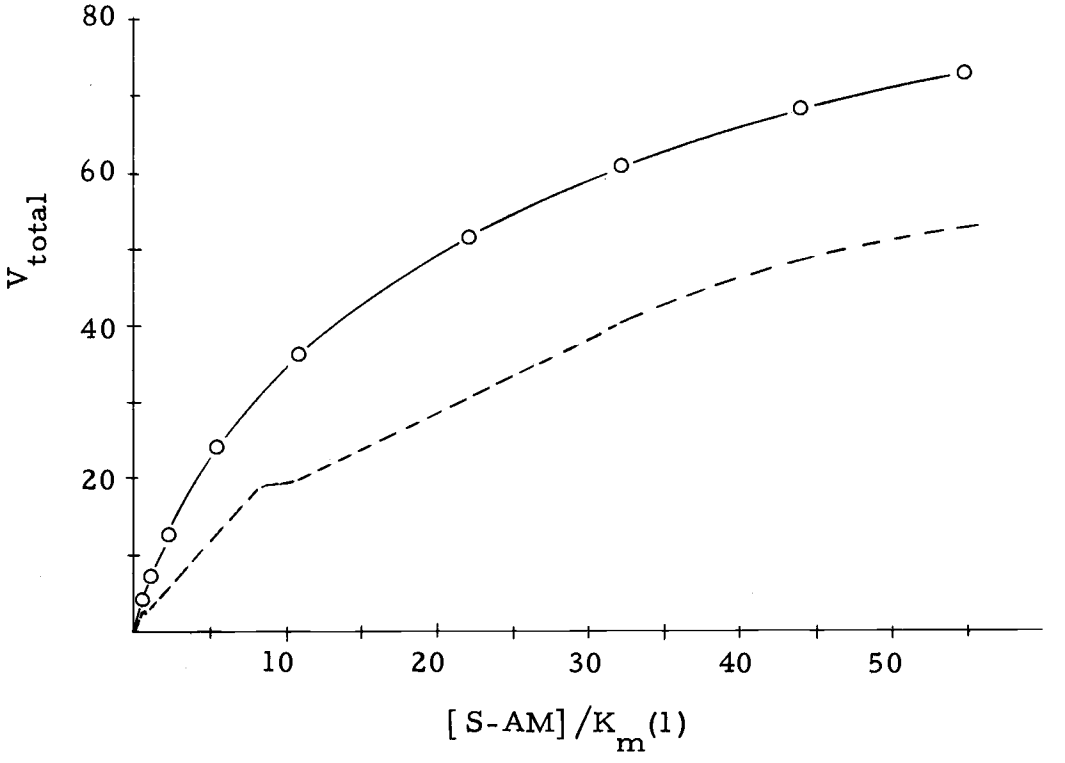


Figure 11. Deviation from the theoretical Michaelis-Menten substrate saturation curve. (o-o), [S-AM]/K_m(1) was varied mathematically and plotted against the resultant velocity as determined from the equation:

$$V_{total} = \frac{V_{max}(1)}{\frac{1+K_m(1)}{S}} + \frac{V_{max}(2)}{\frac{1+K_m(2)}{S}} + \frac{V_{max}(3)}{\frac{1+K_m(3)}{S}}$$

Dotted line represents empirically determined data at different S-AM/K_m(1) values.

Michaelis-Menten curve when the experimentally determined K_m and V_{max} values (Table 5) obtained for S-adenosylmethionine are substituted in the above equation. The dotted line represents observed kinetics at different (S-AM)/K_m(1) values. The differences are quite marked in that the theoretical curve is asymptotic without apparent plateaus. The empirically determined curve is atypical of Michaelis-Menten kinetics and therefore seemingly only could be observed with cooperative effects between the different methyltransferase enzymes and their respective levels of substrate.

Physical separation of the three enzyme activities has been attempted with no success. The enzymes have been completely removed from endogenous sterol substrates through a detergent treatment with subsequent gel filtration and salt fractionation. However, we have been unable to separate the enzymes by further physical methods.

Attempts at differential characterization of the enzyme activities on the basis of the different products and the possible sterol substrates have also been unsuccessful. All three enzyme activities produced fecosterol and ergosterol as the only products. The increased accumulation of ^{14}C-ergosterol was attributed to the presence of more fecosterol at the maximal S-adenosylmethionine levels stimulating a more rapid conversion to ergosterol. The oleate ester of zymosterol was not methylated at any S-adenosylmethionine concentration in our

in vitro system, though the two-fold stimulation of activity by the ester was very interesting. However, until more is known of the role that the sterol esters play in the cell, it is impossible to assign any significance to this observation. It does indicate though, that there is still another binding site on the methyltransferase enzyme(s). Oleic acid by itself severely inhibited the reaction.

The subcellular location of S-adenosylmethionine: Δ^{24}-sterol methyltransferase has been determined. The enzyme has been described previously as a component of the "microsomal" fraction of yeast (54). It now appears that the enzyme is located within the promitochondrial or mitochondrial structures of yeast.

The mitochondrial location of the methyltransferase is attractive considering the amount of published data suggesting a direct linkage between the synthesis of sterols and the acquisition of respiratory competence in yeast cultures (47, 63, 72). Unlike many mitochondrial enzymes, sterol methyltransferase activity is always present in these cultures. The enzyme is not repressed completely during anaerobic growth even though zymosterol is not synthesized during this period (80). Furthermore, the enzyme is not subject to repression by glucose as are most mitochondrial enzymes (33, 49, 87).

The location of the sterol methyltransferase enzyme activities within the mitochondria is not completely clear. Significant amounts of all three activities are found both in the inner membrane and matrix

portions of the mitochondria as evidenced by association with known marker enzymes. Thus the enzymes may be loosely associated with the inner membrane and found in the matrix due to the fractionation procedure, or the enzymes may be located chiefly in the matrix and bind to the inner membrane during isolation. Of importance is the fact that negligible amounts of the enzyme were found associated with the apparent outer membrane portions.

Since the three methyltransferase activities described are located inside of the inner mitochondrial membrane, and they are all similarly inhibited by sodium and potassium, we feel that this may be one method of in vivo regulation of sterol biosynthesis. The experiments with respiring intact mitochondria show a definite effect on sterol synthesis caused by the transport and accumulation of potassium cations in the mitochondria. This type of cation translocation has been reported to be an energy-linked process (67). Because of this, it is feasible to assume that under conditions of active potassium (and probably sodium) transport and accumulation in mitochondria, sterol methyltransferase activity would be decelerated. Since only the methyltransferase enzyme apparently is affected by potassium and sodium in vitro, we feel that this enzyme is a point of regulation for at least the terminal stages of ergosterol biosynthesis.

SUMMARY

The inhibition of S-adenosylmethionine: Δ^{24}-sterol methyltransferase (EC 2.1.1.41) activity by endogenous cellular components has been studied *in vitro*. The principal inhibitors were Na^+ and K^+; Cs^+, NH_4^+ and Li^+ were also shown to inhibit the reaction. The inhibition by potassium cation has been studied, and we have found that energy dependent transport and accumulation of K^+ inhibits the sterol methyltransferase activity in intact respiring mitochondria.

Evidence is presented for three enzymatic activities capable of methylating sterols in cell free extracts of yeast. These differ in their respective Michaelis constants for S-adenosylmethionine, pH optima, and affinity for zymosterol. An apparent cooperative-type relationship with S-adenosylmethionine has been proposed based on observed deviations from theoretical Michaelis-Menten kinetics. However, all three enzymatic activities were similarly inhibited by monovalent cations, had the same subcellular location, and gave identical sterolic products.

The subcellular location of the sterol methyltransferase activity has been determined. All three activities reside in yeast mitochondrial and promitochondrial structures. This provides a direct relationship between ergosterol biosynthesis and respiration since the mitochondria are the site of respiration in the cell. The sterol

methyltransferase deviates from many other mitochondrial enzymes in that it is present at low levels during anaerobic growth and is not subject to catabolite repression by high levels of glucose.

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