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A cell free extract from Saccharomyces cerevisiae containing the enzyme that causes reduction of the 24(28)methylene group on the side chain of ergosterol has been studied. Optimal conditions for the 24(28)methylene reductase were obtained. The enzyme activity was increased in cells grown with ethanol as the substrate. The K_m was calculated as 10 μM. Within the limits of the assay, enzymatic activity was not present in ergostatetraene accumulating mutants that were tested. The enzyme was competitively inhibited when tested against a naturally occurring azasterol. The dissociation constant (K_I) was calculated as 17 μM. The enzyme reaction is not reversible under the conditions provided by the assay.

STEROL 24(28)METHYLENE REDUCTASE IN *SACCHAROMYCES CEREVISIAE*

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To Linda for her love and devotion

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STEROL 24(28)METHYLENE REDUCTASE IN *SACCHAROMYCES CEREVISIAE*

INTRODUCTION

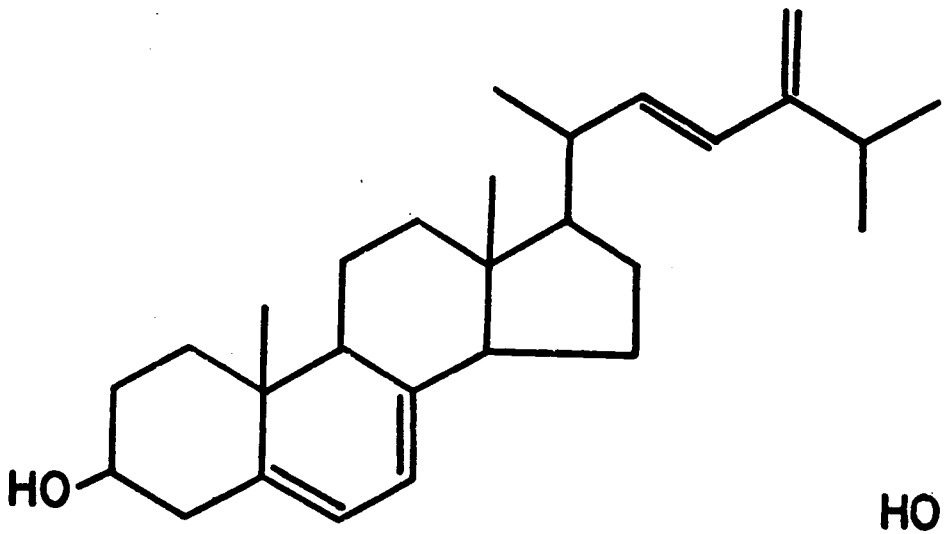
Ergosterol is the predominant sterol found in aerobically growing cultures of wild-type yeast (14). The synthesis of ergosterol closely parallels that of cholesterol up to the formation of lanosterol (12). Both depend on the cyclization of squalene to make up the sterol nucleus (34). However, three unique reactions occur in the lateral chain of the ergosterol molecule that are not present in animal cells. Specifically, they are transmethylation at carbon 24 (reaction A), desaturation at carbon 22 (reaction B), and the reduction of the 24(28)methylene (reaction C) (Figure 1).

Previously reported assay conditions for the 24(28)methylene reductase (19) give extremely low yields and poor reproducibility. With the cell-free conditions reported here, the 24(28)methylene reductase may be easily studied under conditions of high activity. This is important in comparing the synthesis and function of this enzyme under differing physiological conditions.

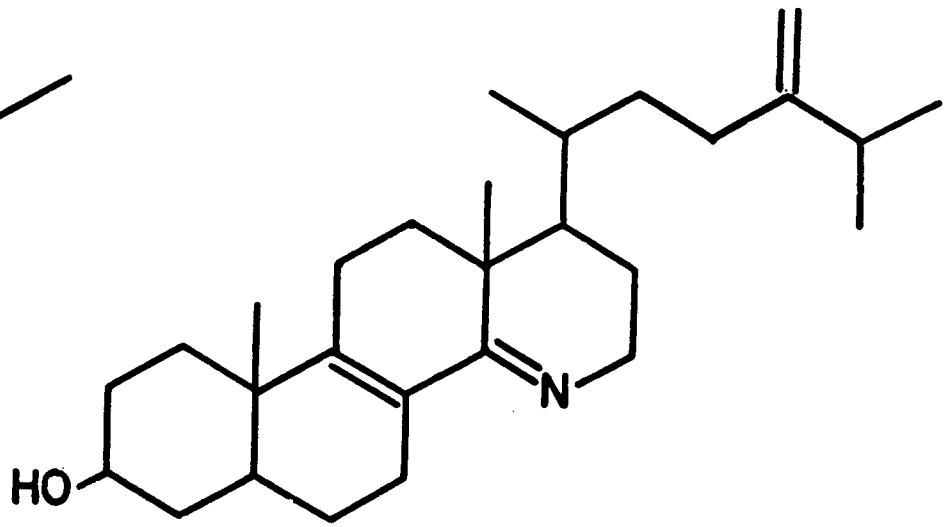
Since among microorganisms these enzymes are specific for fungi, the possibility for selective inhibition of fungal pathogens exists.

Current treatment of some fungal infections involves the use of amphotericin B and other polyenes whose action is nonspecific since they have effects on cholesterol as well as ergosterol (13,26). By developing an *in vitro* assay for one of the side chain modifying enzymes, it would be possible to test the effects of various drugs on the system and thus provide a tool for finding a highly specific means of inhibiting fungal growth.

A naturally occurring metabolite has been isolated from *Geotrichum flavo-bruneum* (9) and has been shown to possess strong antifungal properties (17). This sterol analog (Figure 2) has been identified as 15-aza-24-methylene-D-homocholestadiene (azasterol) (11). Bailey *et al.* (4) have demonstrated that this azasterol causes both *in vivo* and *in vitro* inhibition of the sterol methyltransferase in *S. cerevisiae* (reaction A). Because of the effects of this drug on the methyltransferase, the inhibition of other sterol systems by azasterol derivatives (28,30) and the structural similarity between ergostatetraene and azasterol, the *in vitro* effects of this drug were tested on the 24(28)methylene reductase.



Ergostatetraene



Azasterol

Figure 2

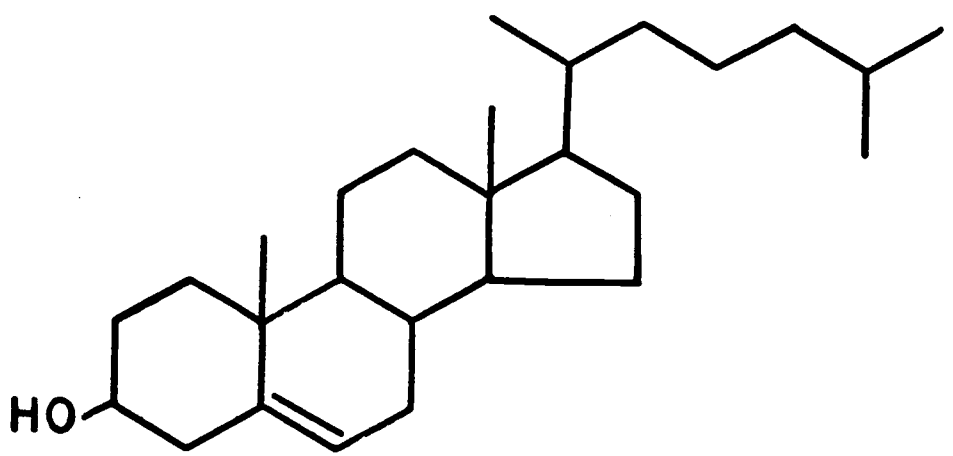
In 1889 Tanret first isolated and accurately described ergosterol (31). The complete structure of the sterol was not elucidated until 1938 (15).

Although both cholesterol (Figure 3) and ergosterol biosynthesis have been extensively studied, most of the work has been done on cholesterol, presumably because of its association with atherosclerosis in mammalian systems. Only in more recent times have the side chain reactions been investigated.

Alexander et al. (1) found that ^{14}C labeled ergosterol could be produced if ^{14}C methyl-methionine was added to cell-free extracts of yeast. They also concluded that the label was added at C-28. Later, Alexander and Schwenk (2) reported that the methyl group from the methyl-methionine donor was transferred intact. Parks (24) conclusively demonstrated that S-adenosylmethione was preferentially used as the source for the C-28 in yeast sterol biosynthesis.

Starr and Parks (29) showed that the rate and extent of methyl transfer increased with aeration time of cultures and was dependent upon the presence of a fermentable carbon source and O_2.

Early work with the transmethylation reaction was hindered by the inability to identify ergosterol precursors, insolubility of sterols in growth medium, and low uptake rates in whole cells. The 24 methyltransferase activity could not be accurately measured, since the whole cell assay systems that were employed depended upon the uptake of methionine, its activation to adenosylmethionine and the synthesis of sterolic precursors.



Cholesterol

Figure 3

Moore and Gaylor (22) prepared a cell-free extract of 24 methyltransferase which showed stoichiometry between the disappearance of zymosterol and adenosylmethionine and the appearance of fecosterol, a methylated zymosterol derivative. This implied that zymosterol was the methyl acceptor and agreed with previous findings that showed that adenosylmethionine played an important role in the methylation reaction. Further studies by Moore and Gaylor (23) suggested that the enzyme had a specific requirement for an acceptor substrate with a demethylated ring structure. They also reported that ergosterol, the end product of fungal sterol synthesis, inhibited the methylation of zymosterol. This indicated that the methyltransferase enzyme might function as a control point in ergosterol biosynthesis through use of a feedback regulation mechanism.

Thompson et al. (32) determined that the subcellular location of the enzyme was within the promitochondrial and/or mitochondrial structures of yeast. A sensitive in vitro assay procedure was used. The 24 methyltransferase was assayed by measuring ^{14}C methyl group incorporation from S-adenosyl(^{14}C Me)methionine into the zymosterol substrate that was added. Homogenized cultures of Saccharomyces cerevisiae were subjected to differential centrifugation, ultra-centrifugation, digitonin precipitation, and sonication in order to separate the mitochondrial matrix, inner membrane, intermembrane, and outer membrane fractions. The mitochondrial location of the enzyme agrees with existing literature that suggests a direct relationship between sterol synthesis and respiratory adaptation in yeast (27).

Thompson also found that in cultures grown in 10% glucose medium, over 95% of the methyltransferase activity was associated with the microsomal fraction (105,000 x g pellet obtained from a 25,000 x g supernatant). When cells were cultured under conditions that favored respiratory metabolism (ethanol medium) less than 2% of the activity was present in the microsomal fraction. Instead the majority of the activity was found in the 25,000 x g pellet (mitochondrial fraction). Since mitochondrial maturation is inhibited in the presence of large concentrations of glucose, an accumulation of promitochondria is found in cultures grown on glucose medium. Using a sucrose density gradient profile and cytochrome oxidase assay procedure, Thompson showed that the microsomal fraction of glucose cultured cells corresponded to the promitochondrial fraction and demonstrated that the methyltransferase enzyme is present in promitochondria. This experiment also indicated that the enzyme is not subject to repression by high levels of glucose. Although a decrease in activity was noticed, the enzyme was always present in the cultures and was not completely repressed during anaerobic growth. By using cyclohexamide, chloramphenicol, and mitochondrial DNA-less mutants (EB-5) and (5015 D), Thompson also showed that the enzyme is synthesized on cytoplasmic ribosomes and coded for in nuclear DNA.

Bailey et al. (6) presented evidence for the presence of more than one methyltransferase. Using an in vitro assay, three enzymic activities were obtained which differed with respect to pH optima, zymosterol affinity, and Michaelis constants for S-adenosylmethionine. Using the same localization procedure as Thompson (32), Bailey found

that all three enzymatic activities occurred in the inner membrane and matrix portion of the mitochondria, with the majority being centered in the matrix portion. Optimal pH values for the three methyltransferases were obtained and reported as 7.1, 7.3, and 7.7. Although no evidence has yet been presented, it is possible that the three enzymes could be found as a methyltransferase complex and function to methylate different sterol substrates. Bailey tested the inhibition of methyltransferase by monovalent cations and found that Na^+, NH_4^+, Cs^+, and Li^+ effectively inhibit the reaction. Na^+ was reportedly the most effective, causing a maximum of 65% inhibition in the presence of 25mM NaCl. Since the enzyme has been shown to be located in the mitochondria, it is possible to consider cation inhibition as a means of regulating sterol synthesis. Pressman (25) has suggested that cation transport through the inner mitochondrial membrane is an energy linked process. With this in mind, it is reasonable to assume that under conditions of active transport of Na^+ or K^+ and their subsequent accumulation in the mitochondria, that methyltransferase activity would be decelerated.

Bailey *et al.* (5) took various strains of bakers yeast that accumulate carbon 27 sterols i.e. strains that lacked the carbon 28, and analyzed them for methyltransferase activity. Both *in vivo* and *in vitro* techniques were employed. No activity was found indicating that the carbon 27 accumulators tested have blocks in the sterol methyltransferase enzyme portion of the pathway. Cholesta-5,7,22,24-tetraene-3β-ol was isolated from cultures of the mutant 8R1 and added as the substrate for the *in vitro* enzyme reaction in place of

zymosterol. The cholestatetraene showed no activity by itself and when included with the normal zymosterol substrate, inhibited the reaction. It would appear that the carbon 22 unsaturated sterols are not suitable substrates for the methylation reaction.

Thompson and Parks (33) showed that the methyltransferase is also effected by the sterol composition of the mitochondrial membrane. Arrhenius kinetics of the enzyme were analyzed in wild-type and sterol mutant strains of yeast. Temperature effects on the enzyme, isolated from 3701-B and nystatin resistant mutants N3 and 3701-BN3 were compared. 3701-B is the wild-type strain and produces ergosterol as its major sterol. N3 and 3701-BN3 produce 8,22 ergostadiene-3β-ol as their major sterol. Transition temperatures indicative of membrane phase change from gel to liquid crystalline were lower in both mutant strains. The substitution of ergosterol by the 8,22 ergostadiene-3β-ol produced a distinct change in the methyltransferase. This might be responsible for the temperature differences between the strains with respect to optimal growth.

Little can be found concerning the 22(23)desaturase enzyme. Currently, there are no published assays for this reaction, (reaction B). Feeding experiments in whole cell cultures of Saccharomyces cerevisiae have presented evidence for the existence of this enzyme. Barton et al. (7) used ^{3}H labeled 7,24(28) ergostadiene-3β-ol as the substrate and isolated labeled 7,22,24(28) ergostatriene-3β-ol. His group also used tritiated 5,7,24(28) ergostatriene and isolated 5,7,22,24(28) ergostatetraene-3β-ol.

Fryberg et al. (16) fed radiolabeled lanosterol to various Candida cultures and isolated labeled 5,7,22 ergostatriene-3β-ol.

Due to the obvious limitations of whole cell enzyme assays, the kinetics and more accurate studies of the 22(23)desaturase will have to wait until an *in vitro* assay procedure is worked out.

Early work that demonstrated the presence of the 24(28) methylene reductase enzyme (reaction C) consisted of feeding labeled 5,7,22,24(28) ergostatetraene-3β-ol (ergostatetraene) into whole cell cultures, and isolating labeled 5,7,22 ergostatriene-3β-ol (ergosterol) (7). The efficient incorporation of the label into the ergosterol indicated that the ergostatetraene was an important precursor of ergosterol.

Crude cell free extracts were first prepared from wet cake-form brewers yeast (19). The 24(28)methylene reductase activity was poor, but Jarman was able to show that NADPH was the essential cofactor of the reduction reaction.

This paper is concerned with the development of a new assay for this enzyme and its inhibition by an azasterol.

Cultures

Saccharomyces cerevisiae strain 3701-B was used for all of the preliminary studies, pH effects, enzyme kinetics, and inhibition studies. Saccharomyces cerevisiae strain NCYC 366, a known ergostatetraene accumulator, was used for the preparation of the enzyme substrate. It was also tested for 24(28)methylene reductase activity. Other strains tested for enzymatic activity were, 317 R50 αRho+, and Candida albicans. Inoculum for the 2-liter flasks used in the experiment was prepared by incubating the organisms in 10 mls of tryptone-glucose broth in a reciprocating shaker at 28°C for 24 hours.

Media and Cultural Conditions

The organisms were routinely grown with shaking in tryptone-glucose broth medium (10). In some instances the 2% glucose was replaced with 2% ethanol (vol/vol) for enhanced mitochondrial development (32). Cells were grown aerobically at 28°C in 2-liter Erlenmeyer flasks containing 500 mls of medium.

Substrate Preparation

^{14}C labeled ergostatetraene [5,7,22,24(28) ergostatetraene-3β-ol] was isolated from S. cerevisiae (strain NCYC 366) grown in 500 mls of tryptone-glucose broth medium supplemented with 10 μCi of [^{14}C] methionine (International Chemical Nuclear Corp.). The cells were harvested via centrifugation and saponified in a 50% methanol, 5% KOH (wt/vol), and 0.5% (wt/vol) pyrogallol solution for 24 hours with refluxing. The nonsaponifiable lipids were extracted with n-hexane, dried under argon, and acetylated in pyridine with acetic anyhydride (3). The acetylated sterols were separated by thin layer chromato-

graphy. Prior to being added as substrate the free-sterol form of ergostatetraene was regenerated by refluxing the acetylated sterol in a 6% KOH methanol solution (wt/vol). The initial purity of the ^{14}C-labeled ergostatetraene was determined by gas chromatography on a Varian aerograph model 2740, using a glass column 2 m long, with a 1-cm outer diameter and a 2-mm inner diameter, packed with 1% OV-17 on 100/120 chromosorb. The column temperature was 275°C and the nitrogen gas flow rate was 20 ml/min. Analysis with a Varian CD 111 integrator indicated that the sample was 98.5% pure. Gas liquid chromatography-mass spectrometric analysis of the labeled ergostatetraene acetate had M^+ at 436 with significant peaks at m/e 376 and 253. Ultraviolet adsorption spectra were performed on a Cary II recording spectrophotometer. Characteristic adsorption peaks were obtained at 232 (λ_{max}), 271, 282, and 294 nm (8). Specific activity of the [^{14}C] ergostatetraene was determined by injecting a sample of known activity into a gas chromatograph. The actual amount was calculated by internal standard analysis using a Varian CDS III integrator with cholestane as the internal standard. Radioactivity of the sample was measured in a Packard Tri-Carb liquid scintillation spectrometer. The specific activity was determined to be 1.65×10^5 cpm/μmole of ergostatetraene. This was used as a substrate throughout the study.

The ^{14}C ergosterol used in the enzyme reversibility experiment was prepared in a manner quite similar to the ^{14}C ergostatetraene. Gas chromatography conditions were identical. Analysis with a Varian CD III integrator showed the sample to be 92% pure. Ultraviolet adsorption spectra as performed on a Cary II recording

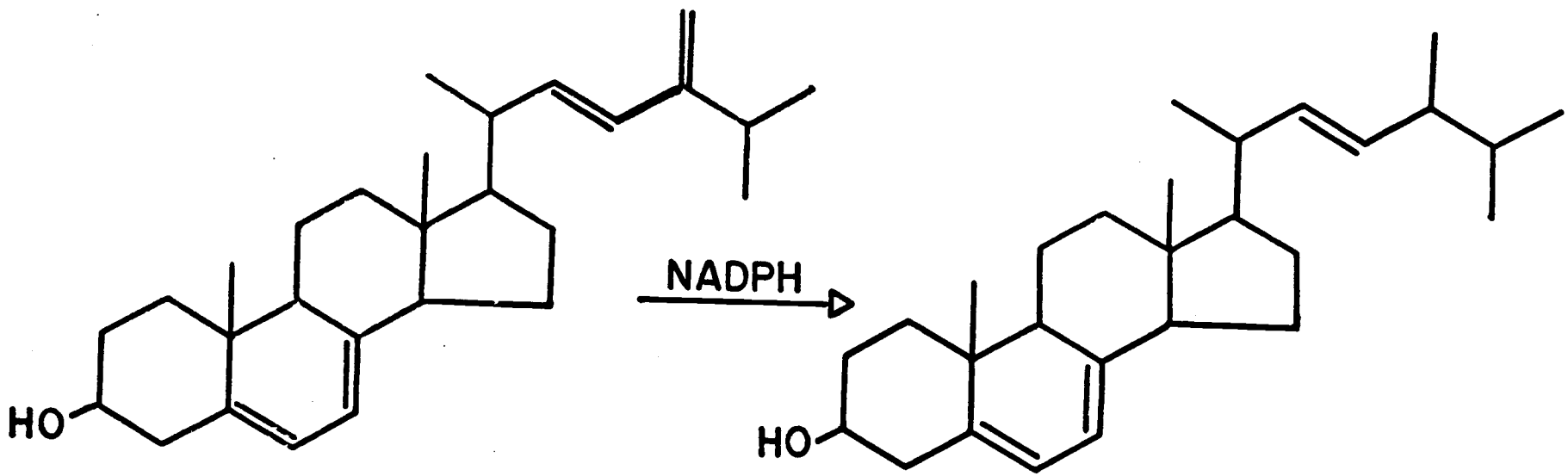
spectrophotometer gave characteristic adsorption peaks at 282 (λ_{max}), and 272 (15). The specific activity was determined to be 8.56×10^4 cpm/μmole.

Enzyme Preparation

Cultures of the organism being tested for enzymatic activity were grown until late exponential phase and then harvested via centrifugation. The cells were washed three times with distilled water and the cell pellet was then resuspended in 1.3M sorbitol, 0.1M Tris (hydroxymethyl)-amino methane (Tris)-hydrochloride, pH 7.5, to a final concentration of 1 gram cells/ml. Ethylenediaminetetra-acetate (EDTA) was added to make the suspension 5mM. In some of the experiments phenylmethylsulfonylfluoride (PMSF) dissolved in 100% ethanol was also added to a final concentration of 1mM. The cell suspension (25 mls) was added to a 75 ml Duran flask and broken with a 45 second burst of a Bronwill MSK tissue homogenizer using 40 grams of 0.25 mm glass beads. All centrifugations following cell breakage were carried out at 5^oC. The cell homogenate was then centrifuged at 2,500 x g for 20 minutes to remove the unbroken cells and other large cellular fragments. This was repeated until pellet formation was no longer observed. The supernatant was then centrifuged at 13,000 x g for 20 minutes. The pellet was washed twice in the same type of buffer and resuspended to a final protein concentration of 19 mg/ml as determined by the method of Lowry (20) using bovine serum albumin as the standard. This served as the standard enzyme fraction for the 24(28)methylene reductase studies.

Enzyme Assay Procedure

The 24(28)methylene reductase activity was measured by adding ^{14}C-labeled ergostatetraene to the enzyme preparation and isolating ^{14}C-labeled ergosterol as the reaction product (Figure 4). The standard reaction mixture used in the assays contained 2.8 mls of 1.3 M sorbitol, 0.1 M Tris-hydrochloride, pH 7.5, 1 µmole of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 1 ml of the enzyme fraction. The ergostatetraene was dissolved in .1 ml of 100% ethanol before being added to the reaction mixture. For the optimal pH determination the tubes were loosely capped and placed in a 28°C incubator with gentle shaking for two hours. The reaction was stopped by the addition of 10 mls of ice cold 5% trichloroacetic acid (TCA). The enzymatic reaction rate was linear over the two hour incubation time that was used. The tubes were placed in an ice bath for 5 minutes and then centrifuged on a model EXD (International Equipment Co.) at 500 x g for 15 minutes. The supernatant was discarded and the sterols were extracted with dimethyl sulfoxide (DMSO), using only slight modifications of the technique of Monner and Parks (21). The sterols were extracted three times with 10 ml aliquots of n-hexane. The hexane was evaporated with gentle heating under N_2 and the sterols were acetylated in pyridine and acetic anyhydride (3). Following the 2 hour acetylation, the excess pyridine and acetic anyhydride were removed by evaporation with heating under N_2. The sterol acetates were then dissolved in diethyl-ether and streaked onto silica gel-60 plates, layer thickness 0.25 mm (E.M. Reagents) that had been impregnated with 10% $AgNO_3$. Redistilled cyclohexane and benzene in a 1:1 (vol/vol) ratio were used as the solvent system to



Ergostatetraene

Ergosterol

Figure 4

separate the ergosterol and the ergostatetraene. Plates were placed in the solvent tanks until the solvent migrated to the top of the plates. They were removed, dried and placed back into the tanks. This was repeated five times. As it was sometimes impossible to monitor the solvent progression constantly, paper wicks were attached to the tops of the plates by means of a separate glass strip and paper clips. This allowed a continuous migration of solvent through the silica gel. Using this method, the plates were kept in the solvent for 18 hours with the wick protruding outside the container to permit the solvent to evaporate from it. With this type of arrangement the ergosterol band moved 2.5 times as fast as the ergostatetraene. For a typical 18 hour migration with wick, the ergosterol band was located approximately 5 cm from the base line. The ergostatetraene band was located 2 cm from the base line. Ergosterol and ergostatetraene standards were spotted on each plate prior to the solvent migration to aid in band identification. After the sterol separation, the ergosterol and ergostatetraene bands were scraped and eluted with diethyl ether through a funnel containing Na_2SO_4. Radioactivity in each fraction was determined by liquid scintillation counting. This was the standard assay procedure used in most of the experiments. For the control tube, 10 mls of 5% TCA were added at time zero to precipitate the protein. Following centrifugation the pellet was stored under argon in the freezer until the rest of the reaction tube sterols were extracted.

In determining the K_m the ergostatetraene substrate concentration ranged from 3 μM to 40 μM. For the optimal pH determination the ergostatetraene concentration in the reaction tubes was 6.5 μM.

During the azasterol inhibition studies the azasterol concentration was tested at 72 μM, 97 μM, and 122 μM. It was dissolved in 100% ethanol and added as .1 ml to each of the experimental reaction tubes. The ergostatetraene concentrations for these studies ranged from 5 μM to 100 μM.

In determining the enzymatic activity in the various mutants and C. albicans the ergostatetraene concentration was 6.5 μM.

For the enzyme reversibility experiments, approximately 24 μg (15 μM) of ^{14}C labeled ergosterol were dissolved in .1 ml of 100% ethanol and added as the enzyme substrate in place of ergostatetraene. For these experiments 1 μmole of oxidized nicotinamide adenine dinucleotide phosphate (NADP) was added in place of the reduced form, (NADPH). All other conditions were the same.

A wide range of enzyme activities was obtained for the various cellular fractions assayed (Table 1). The standard assay procedure was used with 1 ml of the appropriate cellular fraction added as the enzyme.

Cellular fraction assayed	% Ergostatetraene converted to ergosterol	Protein (mg/ml)
500 x g supernatant	16	22
2,500 x g supernatant	16	19
25,000 x g supernatant	7	17
25,000 x g pellet	45	19

The best activity was obtained in the 25,000 x g pellet. This is similar to the results reported for the methyltransferase (6). The lowest activity was found in the 25,000 x g supernatant.

Various co-factors were tried (Table 2) but NADPH gave the best conversion as had been previously reported (19). Each of the tubes in Table 2 contained 1.3M sorbitol, 0.1M Tris (hydroxymethyl)-amino methane (Tris)-hydrochloride, pH 7.5, [^{14}C] ergostatetraene dissolved in .1 ml 100% ethanol and 1 ml of the standard enzyme fraction.

Additives	% Ergostatetraene converted to ergosterol
Tris-hydrochloride buffer	3
NADH	4
ATP	4
PMSF	5
Dithiothreitol	6
NADPH	10
NADPH + EDTA	53

Reduced nicotinamide adenine dinucleotide (NADH) appeared to be inactive as a coenzyme. Neither adenosine 5'-triphosphate (ATP) nor

dithiothreitol provided significant stimulation alone. Phenylmethyl-[19] sulfonylfluoride, (PMSF) also failed to provide any increase in activity. EDTA is important in order to obtain optimal enzyme activity. When EDTA was used in conjunction with NADPH, up to a five fold increase in reductase activity was observed. There was no significant change in enzyme activity when EDTA was added to reaction mixtures using NADH, ATP, dithiothreitol, or PMSF.

To determine if an increase in ergostatetraene accumulation might correlate with a decrease in 24(28)methylene reductase activity, two ergostatetraene accumulators NCYC 366 and 317 R50 αRho^{+}, were grown and used for enzyme preparation. Within the limits of the assay, neither organism displayed enzymatic activity.

Candida albicans was also tested. Originally, the organism was to be used in an in vitro 24(28)methylene reductase azasterol inhibition study. However, in vitro activity was not obtained. This might be due to improper assay conditions since there is a significant difference in Saccharomyces and Candida physiology. According to a previously published paper (16) the most likely substrate for the 24(28)methylene reductase in Candida, is a 7,24(28) ergostadiene 3β-ol. If this is correct then it is possible that the ergostatetraene would not be a suitable substrate in the Candida system.

In order to develop the best possible conditions for the assay it was necessary to determine the optimal pH. Assays were performed with buffers ranging from pH 6.0 to pH 8.5 (Figure 5). Both Tris (hydroxymethyl)-amino methane (Tris)-hydrochloride and K_2HPO_4 buffers were used. The optimal pH centered on 7.5 and is about the same as the reported optimal pH of the methyltransferase (23).

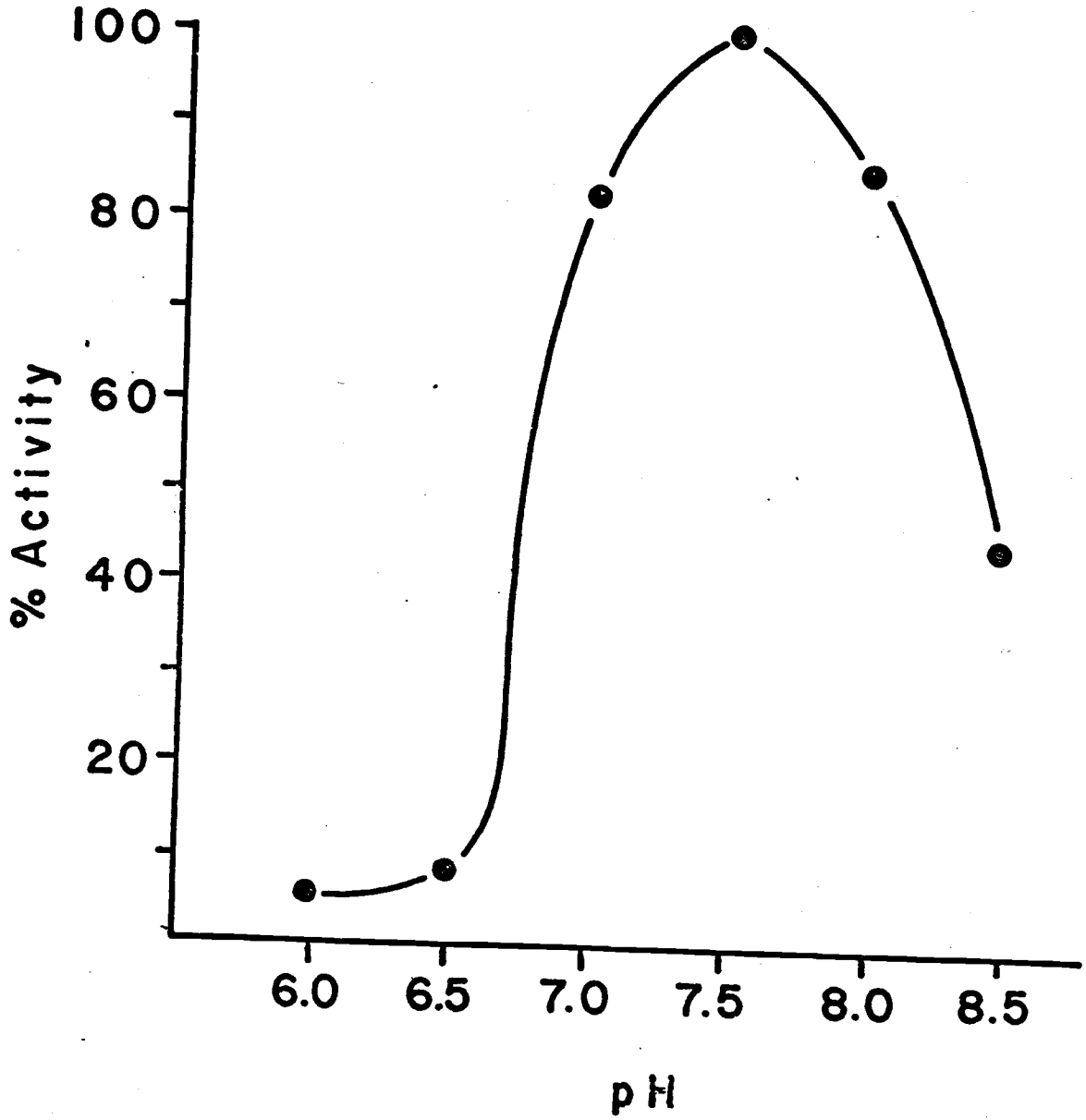


Figure 5

Lineweaver-Burk plots were drawn and the apparent K_m obtained from this set of experiments was about 10 μM. This indicates a rather strong enzyme affinity for the substrate and is unique since the substrate is water insoluble. Two different plots are shown on the graph (Figure 6) the lower curve is for cells cultivated on ethanol, whereas the upper curve is for glucose grown cells. Higher activity in ethanol-grown cells is consistent with the results of earlier experiments that supported a role for ergosterol in the maintenance of respiratorial competency in these organisms (L. W. Parks, R. B. Bailey and E. D. Thompson, Proceedings of the Symposium on Adenosylmethionine, in press).

When [^{14}C] ergosterol was used as the substrate and NADP as the hydrogen acceptor little or no label was incorporated into ergosta-tetraene. Under these assay conditions it appears that, at least in vitro, the 24(28)methylene reduction is not a reversible step.

When azasterol was included in the reaction mixture of the standard in vitro assay, a drastic decrease in 24(28)methylene reductase activity was observed. Azasterol concentrations of 72 μM, 97 μM, and 122 μM all caused a dramatic inhibition of enzyme activity. Lineweaver-Burk plots were drawn (Figure 7). The figure shows competitive inhibition; which is the same type observed for azasterol induced inhibition of the yeast methyltransferase (4). The competitive nature of the inhibition is consistent with in vivo experiments that demonstrated that azasterol induced inhibition is of a reversible nature (18). The average dissociation constant (K_i) calculated for the compound was 17 μM.

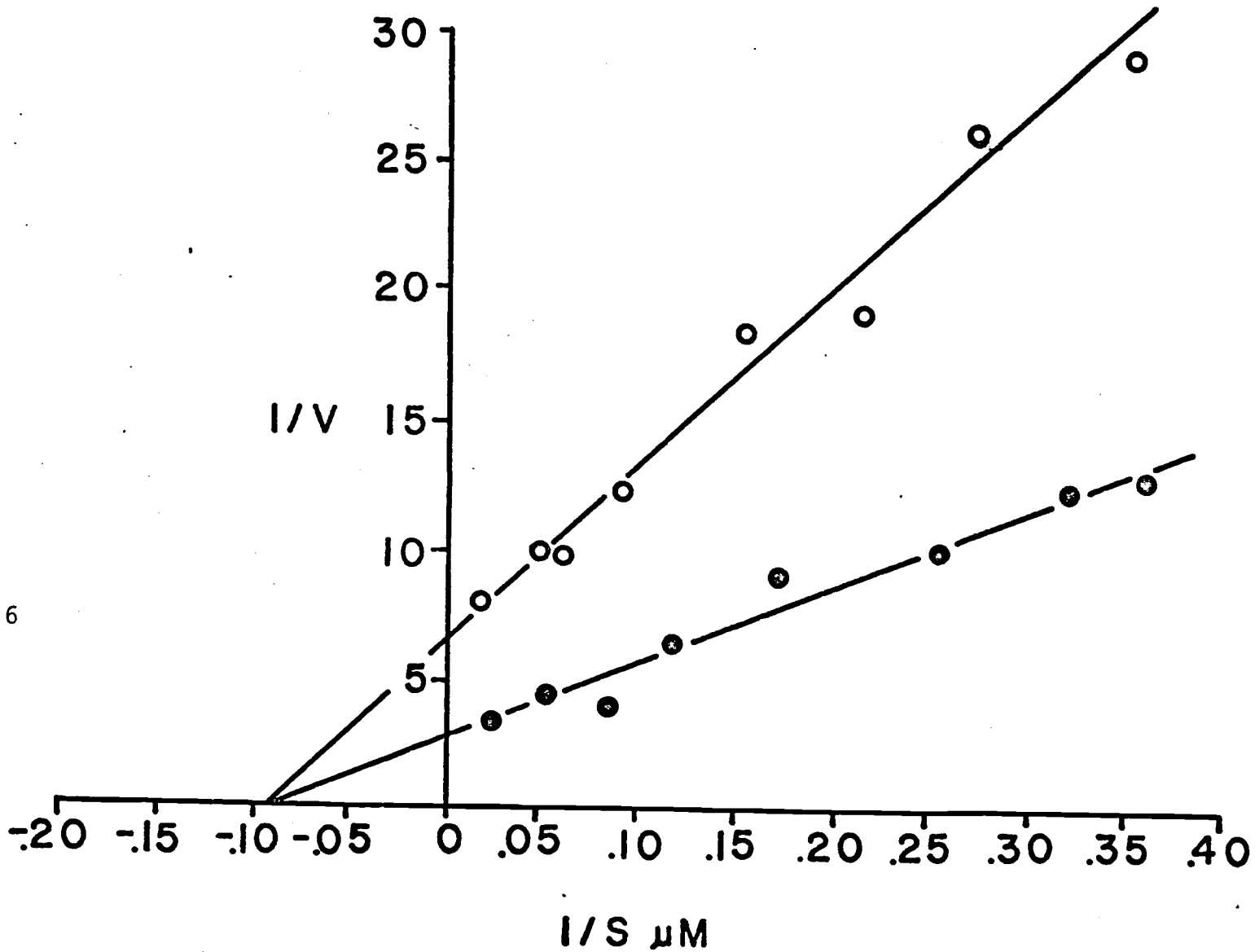


Figure 6

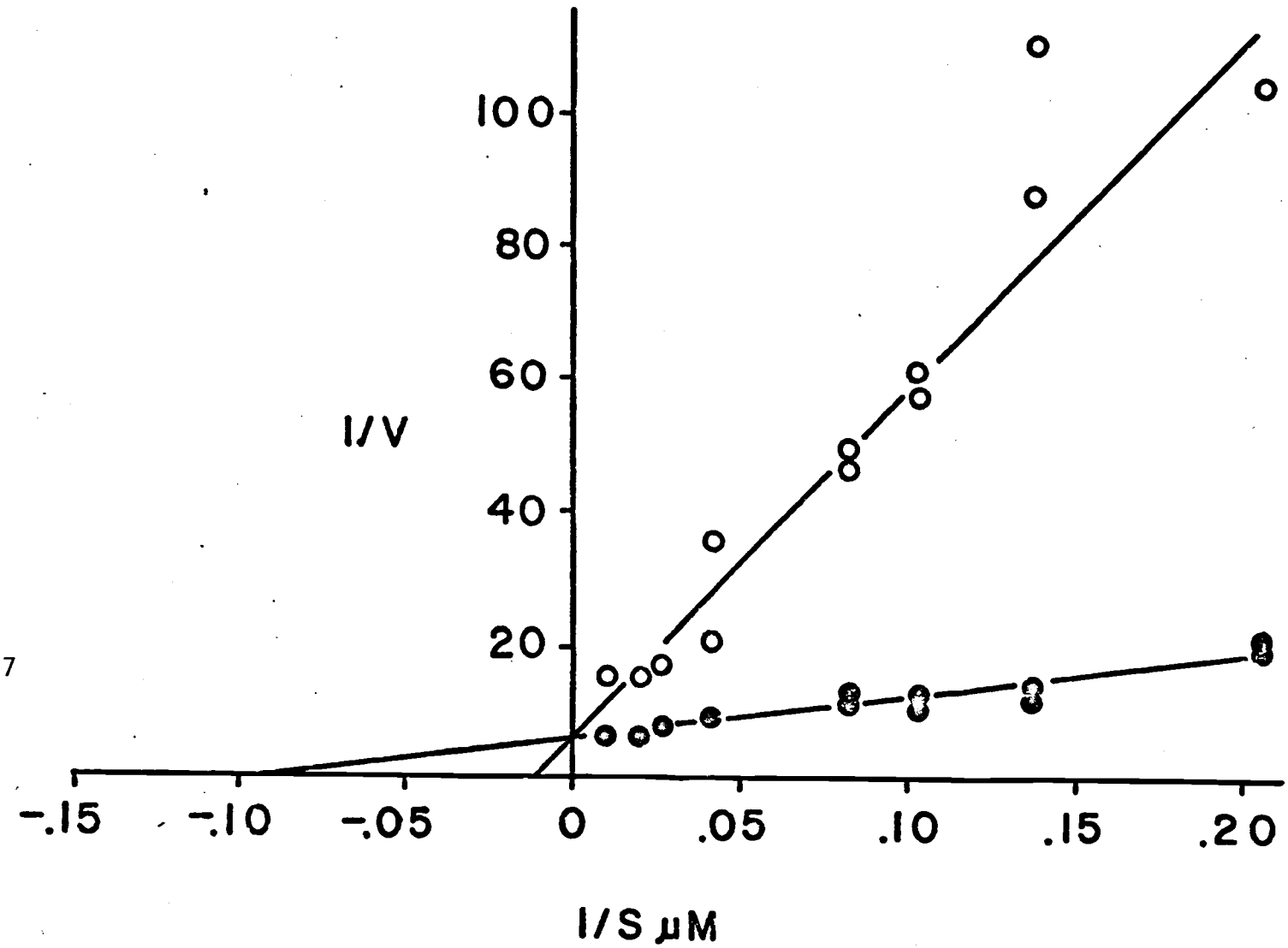


Figure 7

The unique reactions involved with side chain modifications are important in understanding the role of sterols in cells. By selectively controlling these reactions it might be possible to develop a drug that would be extremely specific in its mode of action and highly effective against fungal pathogens.

Control of these enzymes should also lead to the accumulation of intermediates in the sterol pathway and might allow one to determine the order of sterol synthesis leading to the formation of ergosterol. The accumulation of sterols other than ergosterol would allow one to better understand the physiological role of sterols in fungi.

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