AN ABSTRACT OF THE THESIS OF

JAN ROSS TURNER (Name) for the Ph. D. (Degree) in MICROBIOLOGY (Major)

Date thesis is presented May 11, 1965

Title THE ROLE OF S-ADENOSYLMETHIONINE IN THE BIOSYNTHESIS OF ERGOSTEROL AND RELATED STEROLS IN SACCHAROMYCES CEREVISIAE

Abstract approved Redacted for Privacy
(Major professor)

An enzyme system was prepared from Saccharomyces cerevisiae, strain MCC, which carries out the transfer of the methyl group from S-adenosylmethionine or methionine to the side chain of yeast sterols. The cell-free system has been shown to be incapable of synthesizing ergosterol but carries out the synthesis of two as yet unidentified sterols which contain the methyl group of S-adenosylmethionine.

Whole cells were shown to synthesize both ergosterol and the two sterols found in the cell-free system. By time course studies of pulsed methionine-methyl-C^{14} incorporation and by feeding experiments, it has been possible to show that the two sterols synthesized by the cell-free homogenate are precursors of ergosterol.

The cell-free system requires for maximal activity ATP, $MnCl_2$, K_2CO_3, ascorbate, acetate and glucose-6-phosphate. When S-adenosyl-methionine is the methyl donor, ATP is not necessary. The function of K_2CO_3, since it is not incorporated into sterol to any extent,

was tested as a possible allosteric effector for this enzyme. Neither heating nor treatment with $HgCl_2$ produced a preparation which was insensitive to carbonate.

Evidence was found that the two precursors have a double bond at C-22, as does ergosterol, but differ in the unsaturation of the ring system.

THE ROLE OF S-ADENOSYLMETHIONINE IN THE BIOSYNTHESIS OF ERGOSTEROL AND RELATED STEROLS IN SACCHAROMYCES CEREVISIAE

by

JAN ROSS TURNER

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1965

APPROVED:

Redacted for Privacy

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Date thesis is presented May 11, 1965

Typed by Ruth Baines

ACKNOWLEDGMENTS

I want to express my appreciation to Dr. L. W. Parks for his uncompromised effort and guidance in this investigation, which often prevented it from traveling up many a well-known cul-de-sac.

I also wish to thank the staff and graduate students of the Department of Microbiology with whom I have spent so many hours at Oregon State.

Gratitude is expressed to the Davidson Chemical Co., Baltimore, Maryland for the gift of silica gel and to Peter D. Klein, Argonne National Laboratories, for making available data in advance of publication.

TO MARLENE

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THE ROLE OF S-ADENOSYLMETHIONINE IN THE BIOSYNTHESIS OF ERGOSTEROL AND RELATED STEROLS IN SACCHAROMYCES CEREVISIAE

INTRODUCTION

The role of methionine in protein synthesis has been well established for many years, however recently it was found that methionine also serves an important function in the transfer of methyl groups, i.e. transmethylation. Methionine participates in transmethylation reactions through the intermediate S-adenosylmethionine (AM) which is formed from ATP and methionine. Cantoni recently has determined that in at least forty different reactions AM functions as a methyl group donor, indicating that this process is widely utilized by biological systems.

Among these forty reactions is the one in which the methyl group of AM is transferred to the 24th carbon atom of ergosterol. Although transmethylation from AM to nitrogen, sulfur, and oxygen atoms had been shown, this was the first instance in which a new carbon-carbon bond was formed as a result of the transfer of a methyl group originating from AM.

The biological importance of ergosterol has been of great interest, since it was discovered that ergosterol would serve as a source for vitamin D after irradiation with ultraviolet light. The biosynthesis of ergosterol has not been afforded much intensive study. The propensity of S. cerevisiae and other fungi to synthesize large amounts of sterol and consequently to commit large amounts of energy

and carbon to this process suggests an important role for sterol in the metabolism of these organisms. However, as in the case of mammalian sterols, the metabolic function of yeast and fungal sterols has not been clearly determined.

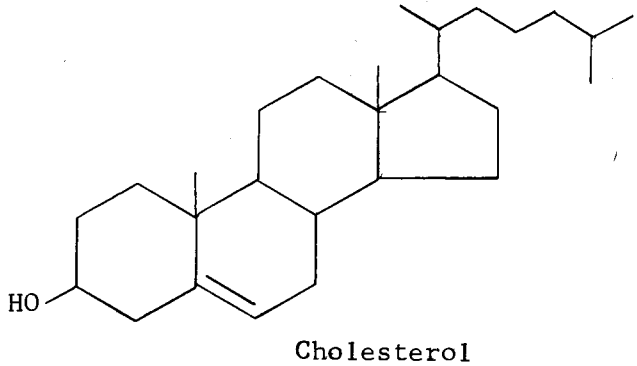
A very useful approach to the study of the biosynthesis of ergosterol has been found because of the fact that AM functions to supply one of the carbon atoms of the molecule. AM contributes only the 28th carbon atom of ergosterol, all other atoms being derived preferentially from acetate. Using AM-methyl-C^{14} it has been found that the terminal sequence of reactions leading to ergosterol may be followed by tracing the course of the radioactive methyl group in whole cell and cell-free systems. By use of this method it has been possible to gain some insight into the biosynthesis of ergosterol and some related sterols in yeast.

REVIEW OF LITERATURE

Sterols are secondary alcohols having 27 to 30 carbon atoms and melting in the range of 100 to 200°C. The major sterol found in animal systems is cholesterol, whereas in yeast, many other fungi, and some plants ergosterol is the major sterol.

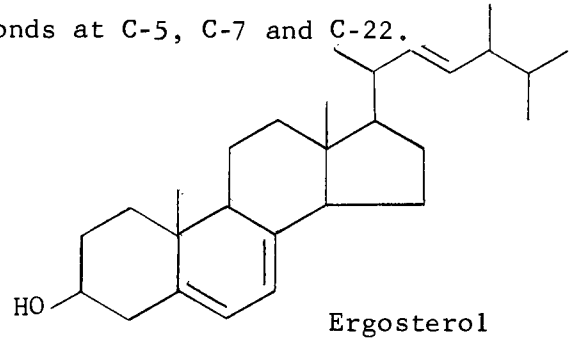
Chevreul, in 1812 (Fieser, 1959) differentiated saponifiable from non-saponifiable material in animal lipids and is given credit for discovering cholesterol. Subsequently, cholesterol was found to be the major constituent of gallstones and this became, and remains today, one of the major sources of commercial cholesterol. Ergosterol was isolated from ergot in 1889 by Charles Tanret. In 1920, Smedley-Maclean isolated and purified a sterol from yeast which she suggested to be identical to ergosterol. Yeast have proven to be an abundant source of ergosterol, since it constitutes approximately two percent of the dry weight of the cell.

The structure of cholesterol was established about 1915 as a result of the independent efforts mainly of Adolf Windaus and Otto Diels (Fieser, 1959). The structural formula assigned is shown below.



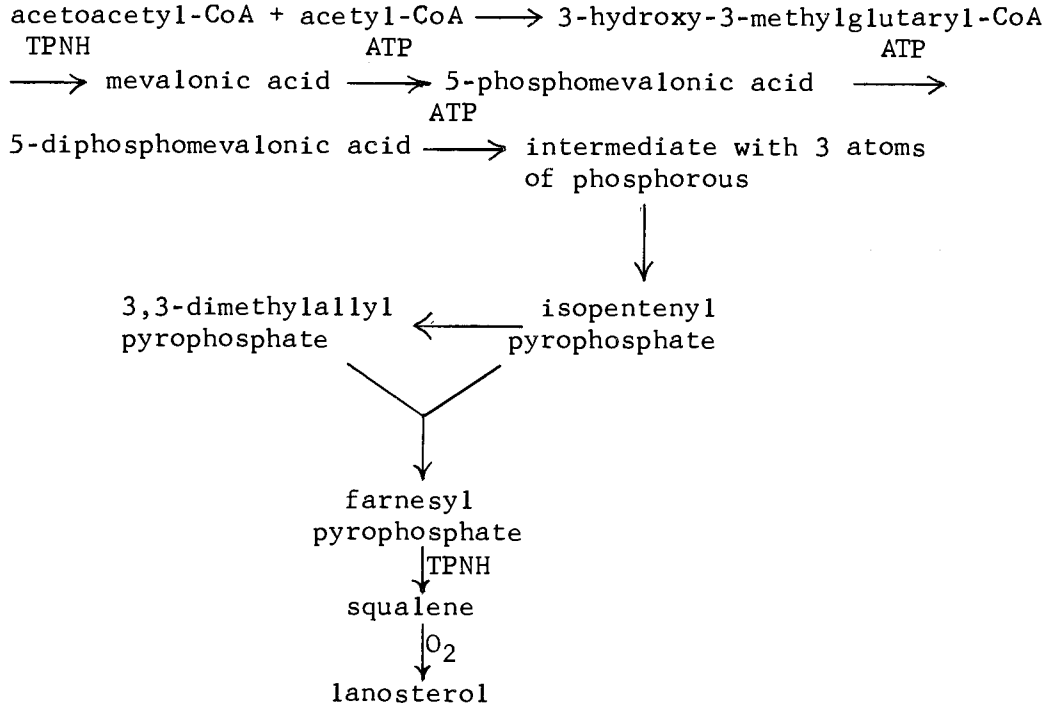
Cholesterol

The skeletal structure of ergosterol was established in 1933 by Chuang, and it was subsequently shown to be a 28-carbon sterol having double bonds at C-5, C-7 and C-22.



Ergosterol

The biosynthetic reactions leading to the synthesis of lanosterol, an intermediate common to both ergosterol and cholesterol, have been found to follow parallel courses in both yeast and mammalian systems (Cornforth, 1959). Enzymes catalyzing identical reactions have been found in cell-free preparations from both systems. The reactions of this sequence are as follows:



As was shown by Tchen and Bloch (1956), molecular oxygen is required for formation of lanosterol from squalene in rat liver homogenates. When the reaction was run in the presence of O_2^{18}, the lanosterol formed contained O^{18}. Although similar experiments involving O_2^{18} have not been attempted in yeast, it can be assumed that the reaction is similar to that of the mammalian system. This is borne out by the molecular oxygen requirement for sterol synthesis to occur in yeast (Klein, 1955).

The biosynthetic reactions which occur subsequent to lanosterol, formation and leading to cholesterol are fairly well delineated. However the sequence of reactions leading from lanosterol to ergosterol have not been well studied and are for the most part unknown.

Klein (1955), has demonstrated that yeast grown anaerobically do not synthesize sterols but accumulate large amounts of hydrocarbon, presumably squalene. On aeration of these hydrocarbon-rich yeast, the sterol content increases from three to eight fold. Starr (1962), has shown that the rate of sterol synthesis under these conditions reaches a maximum two hours after aeration has begun. Yeast, therefore, constitute a system in which sterol synthesis can be easily regulated and consequently one which is ideal for the study of ergosterol synthesis.

As indicated previously, acetate can serve as the major carbon source for both ergosterol and cholesterol (Clayton and Bloch, 1956). Klein and Booher (1955) have shown that cell-free yeast homogenates incubated with adenosine triphosphate and C^{14}acetate will incorporate

radioactivity into the non-saponifiable fraction. In 1953, Hanahan and Wakil degraded the side chain of ergosterol synthesized from yeast grown in carboxyl labeled acetate. They established that the C-24 methyl group did not contain a significant amount of radioactivity. A similar situation was shown to occur in the biosynthesis of the C-20 steroid, eburicoic acid, by Penicillum sulfureus (Dauben, Fonken and Boswell, 1957). In this instance, however, it was indicated that formate serves as the source of the side chain methylene group. When this organism was grown in the presence of sodium formate-C^{14}, 48 times as much radioactivity was found in the C-28 carbon as in any of the other carbon atoms of the molecule. Formate was also shown to be a very efficient source of carbon for the C-28 methyl group of ergosterol by Danielsson and Bloch (1957). They demonstrated that the C-28 carbon of ergosterol accounted for, within experimental limits, all of the radioactivity incorporated into ergosterol from C^{14}formate. It was also shown that no radioactivity was incorporated into squalene, zymosterol, or lanosterol indicating that the C-28 carbon probably is added at a late state of synthesis and to a preformed sterol nucleus.

In 1957, Alexander, Gold and Schwenk performed experiments with cell-free yeast homogenates to test the efficiency of bicarbonate, formaldehyde, propionate and methionine as sources for the C-28 carbon of ergosterol. They found that incorporation of radioactivity into the non-saponifiable fraction was 20 percent for methionine-methyl-C^{14}, 1.2 percent for acetate-1-C^{14}, 1.0 percent for sodium bicarbonate, 0.4 percent for formaldehyde and 0.5 percent for propionate

(1 or 2)-C^{14}. After degradation of the side chain of ergosterol synthesized from labeled precursors, it was found that methionine contributed 20-30 times as much C^{14} into C-28 as to any other carbon atom of the molecule; whereas, acetate carbon was almost evenly distributed between the side chain carbons and the nuclear carbons. This indicated a specific donation of the methyl group to the side chain from methionine. It was also noted that squalene but not zymosterol is converted to ergosterol by yeast homogenates.

In a second communication Alexander and Schwenk (1957) demonstrated by dilution experiments that methionine-methyl-C^{14} was a more efficient source of the C-28 carbon of ergosterol than formate or serine. In addition it was shown that aminopterin, a folic acid antagonist, decreased incorporation of radioactivity from formate but not from methionine-methyl-C^{14}. This suggested that formate was being first converted to methionine before incorporation into sterol.

To substantiate the fact of transmethylation from sulfur to carbon, a mixture of methionine-methyl-C^{14} and methionine-methyl-H^3 was incubated with a cell-free yeast homogenate. After degradation of the side chain of ergosterol and isolation of the C-28 carbon atom, the ratio of the H^3/C^{14} was found to be the same as in the added methionine, indicating that the methyl group was transferred intact.

It was established in 1947 (Borsook and Dubnoff) that in order for methionine to participate in certain methyl group transfers it must first be activated by reaction with adenosine triphosphate. S-adenosylmethionine was later shown to be the product of this

activation reaction (Cantoni, 1951). Subsequently, in 1958, Parks demonstrated by dilution experiments utilizing a cell-free yeast homogenate that S-adenosylmethionine was a more efficient donor of the methyl group than methionine for ergosterol synthesis.

The transfer of the methyl group of methionine to the side chain of ergosterol through the intermediation of S-adenosylmethionine represents the first sulfur to carbon transmethylation demonstrated. However that the methyl group is transferred intact is now questionable since Jauréguiberry et al (1964, 1965) have shown by mass spectrographic examination of ergosterol synthesized from methionine-methyl-d_3 that only two of the deuterium atoms can be detected in the ergosterol produced. This is similar to the reaction in which tuberculostearic acid (10-methyl stearic acid) is synthesized by a methyl group transfer to a double bond of oleic acid (Lennarz, Scheuerbrandt and Bloch, 1962) which has also been shown to retain only two of the deuterium atoms in the product (Jauréguiberry et al., 1964, 1965). Jauréguiberry et al. (1965) assume that this reaction, as in the ergosterol transmethylation, is mediated by S-adenosylmethionine and that in both transmethylation reactions there is probably a common intermediate in which one hydrogen is lost from the transferred methyl group. However in neither of these cases has an intermediate been isolated which would substantiate this postulate.

A number of minor sterols have been isolated from yeast (Fieser, 1959) however, none of these have been shown to be a precursor of ergosterol. The present state of knowledge of ergosterol biosynthesis

subsequent to lanosterol formation then is: zymosterol, although the second major sterol found in yeast is not a precursor of ergosterol; carbon 28 is derived from methionine through S-adenosylmethionine by a reaction at a late stage in the synthetic sequence in which one hydrogen of the methyl group is lost; and the remaining carbons of the molecule are derived from acetate.

MATERIALS AND METHODS

Cultures

A wild type diploid strain of Saccharomyces cerevisiae, MCC was used for all experiments. All stock cultures were kept at 5°C and transferred once each month. Inoculum for the 2 liter flasks was prepared by aerating strain MCC in 50.0 ml of yeast complete medium (YCM) at 30°C for 12 hours.

Media

A medium designated YMAF was used to grow large amounts of cells. The composition is as follows: 1% yeast extract, 2% glucose (autoclaved separately), 0.1% ammonium chloride, 1.1% K_2HPO_4 and 1.85% KH_2PO_4. Seventeen hundred ml of the medium (minus the glucose) was placed in a 2 liter flask and autoclaved. Immediately after autoclaving, the glucose was added and the flasks allowed to cool before inoculation.

Aeration medium - 1% glucose, 0.1 molar KH_2PO_4

Yeast complete medium (YCM) - 2% glucose, 1% yeast extract and 2% tryptone

Intermediates and substrates

S-adenosylmethionine (AM) was synthesized from l-methionine and adenosine triphosphate (ATP) using a rabbit liver preparation according to the procedure of Cantoni (1957) but incorporating the modification of Bremer and Greenberg (1961). S-adenosylmethionine was isolated

as the crystalline tri-reineckate, recovered by dissolving the complex with 2-butanone and extracting the reineckate with ethyl ether. Radioactive S-adenosylmethionine was prepared using l-methionine-methyl-C^{14} in the reaction.

All other substrates and intermediates were obtained from commercial sources at the highest available purities.

Growth conditions for cells

To obtain maximum sterol production the cells were grown according to the anaerobic-aerobic technique of Klein (1955). At the end of the anaerobic incubation period the cells were harvested by centrifugation, washed twice with 0.1 molar phosphate buffer (pH 6.5) and suspended in the aeration medium. This suspension (approximately 14 mg dry wt. cells/ml) was aerated by shaking on a rotary shaker for 2 hours at 30°C. The cells were then re-harvested, if to be used for cell-free experiments, or incubated further with additional substrate when used for whole cell experiments.

Whole cell experiments

To study sterol synthesis in whole cells, the anaerobically grown cells were aerated for 2 hours in the aeration medium. At the end of this period methionine-methyl-C^{14} was added and aeration continued for specified intervals. The cells were then harvested by centrifugation, washed twice with distilled water, saponified and the sterols extracted.

For incubation of whole cells with isolated sterols, the sterols were dissolved in petroleum ether (bp 30-60°C) and placed in a 250 ml Erylenmeyer flask. The solvent was then evaporated with a stream of nitrogen. The cells were suspended in 50 ml of the aeration medium and placed in the flask. Incubation was carried out for 5 hours at 30°C in a Dubnoff incubator-shaker. The cells were then harvested by centrifugation, saponified and the sterols extracted.

Preparation of cell-free enzyme system

Aerated cells were ground in dry ice according to the procedure of Klein (1957). This was followed by centrifugation for two ten minute periods at 1500 x g to remove the unbroken cells and cellular debris. Two ml portions of the supernatant fluid were then passed through an eight ml G-50 Sephadex column. The extract after Sephadex treatment was termed the cell-free enzyme and was used in all subsequent cell-free experiments.

Cell-free reaction conditions

For preparation of sterols or determination of reaction components a cell-free sterol synthesizing system was constructed as follows: 1.5 ml of the cell-free enzyme, 60 μm ATP, 20 μm glucose-6-phosphate (G-6-P), 20 μm potassium ascorbate, 120 μm potassium acetate, 4 μm $MnCl_2$, 60 μm K_2CO_3, 0.2 μm (1 μc) methionine-methyl-C^{14}, or a specified amount of S-adenosylmethionine-methyl-C^{14} was added to a glass stoppered tube in a total volume of 3.2 ml. The tubes were

then stoppered and incubated for 5 hours at 30°C. At the end of the incubation period 1.0 ml of 40% KOH was added to stop the reaction. Zero time controls were obtained by adding the alkali prior to incubation.

Isolation and analysis of sterols

Sterols were isolated from both the cell-free system and whole cells by saponification with potassium hydroxide followed by extraction with three 10.0 ml portions of petroleum ether (bp. 30-60). In the cell-free system unless otherwise specified, 1.0 mg of carrier ergosterol, 10.0 ml 95% ethanol and a boiling chip were added to each tube. The tubes were then heated in a water bath at 80°C for 20 minutes. The non-saponifiable fraction was extracted and transferred to scintillation vials for counting or prepared for chromatography. For isolation of sterols from whole cells 2.0 ml of a suspension of cells, 1.0 ml 40% potassium hydroxide and a boiling chip were added to a glass stoppered tube. The tubes were loosely covered with foil and placed in a steamer for six hours. The preparations were then cooled and the sterols extracted.

To analyze for C^{14}, the sterols in petroleum ether were transferred to scintillation vials, the solvent evaporated under nitrogen and 20.0 ml of scintillation fluid (3.0 gm 2-5-diphenyloxazole (PPO) plus 0.1 gm 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) per liter of toluene) added. The samples were counted in an automatic, model 314-DC Tri-Carb Liquid Scintillation Spectrometer. For the fractions

isolated via column chromatography the sterols were analyzed by C^{14} counting and by Liebermann-Burchardt color (Fieser, 1959). One half of each fraction or every third fraction in some cases was removed to a scintillation vial for counting, the remaining half was evaporated to dryness under nitrogen and 3.0 ml of chloroform added. To each sample in chloroform 2.0 ml of a solution of 5% concentrated sulfuric acid in acetic anhydride (v/v) was added, the tube shaken and placed in an ice bath. Optical density measurements were taken exactly 15 minutes later at 625 mμ with a Beckman DU spectrophotometer.

Digitonin precipitation of sterols

Digitonin, a saponin, is known to react with 3- -hydroxy sterols. Compounds not precipitating with digitonin could not, however, be termed non-sterolic because the 3-alpha-hydroxy sterols do not form a digitonin complex. These compounds were therefore termed simply nondigitonin precipitable materials.

When it was desired to precipitate the sterols as digitonides, the sterols, in solvent, were placed in a scintillation vial, 1.0 mg carrier ergosterol added and the solvent evaporated. The sterols were dissolved in 4.0 ml of 1:1 acetone:absolute ethanol and 4.0 ml of a solution of 0.5% digitonin in 50% ethanol added. The samples were refrigerated overnight followed by centrifugation at 2500 rpm in an International model EXD centrifuge for 15-20 minutes. The precipitate was then washed twice with 5.0 ml portions of 1:1 actone:ether and twice with 5.0 ml portions of ether. The washed precipitate was dried at

55°C for 30 minutes.

To liberate the sterols from the digitonin complex the digitonide was dissolved in pyridine and heated at 80°C for 30 minutes. Ether was then added to precipitate the digitonin and extract the sterols.

Chromatography of sterols isolated from yeast

The column was constructed of a 100 cm x 0.45 cm glass tube fitted with a 2000 ml reservoir at the top and provided with an attachment to apply positive pressure (approximately 5-7 lb/sq in) to the top of the column. The column was packed with silica gel #12, mesh 325-200 (Grace Chemical Co.). In this procedure it was necessary to form acetate derivatives of the sterols before separation (Klein, 1962).

Fractions from columns were collected with an LKB automatic fraction collector. To reduce evaporation, the effluent was collected directly into the tubes rather than through a collecting syphon.

Thin layer chromatography of sterols was carried out according to the procedure of Avigan, Goodman and Steinberg (1963). Sterols were detected on the thin layer plates by spraying with Liebermann-Burchardt reagent followed by heating at 55°C for 20 minutes. Radioactivity of sterols separated by thin layer chromatography was determined by scraping the spot off of the chromatoplate and suspending the silica gel in scintillation fluid in a counting vial. The silica gel settles to the bottom of the vial while the sterols dissolve in the toluene counting solution.

RESULTS

Cell-free yeast homogenates capable of sterol synthesis have been used in the study of the transmethylation reaction in ergosterol synthesis, (Parks, 1958; Alexander and Schwenk, 1957; Alexander, Gold and Schwenk, 1957). However the cofactor requirements of these systems were not investigated extensively.

In this investigation it has been found that in order to get good cell breakage and an active enzyme preparation, the method of Klein (1957) was most satisfactory. This consists of grinding the cells in a mortar after freezing in dry ice. Various other methods of cell breakage such as sonication, dried cell preparations, and toluene disruption give either poor cell breakage or inactive enzyme preparations.

A series of experiments was conducted to determine what cofactors would produce the greatest amount of incorporation of radioactivity into the non-saponifiable fraction. The effects of divalent cations, reducing substances and various cofactors were tested. Further data supporting those experiments are summarized in tables 1 and 2. Either methionine or S-adenosylmethionine may be used as substrate for this transmethylation reaction since yeast have been shown to contain a highly active AM-synthetase system (Pigg, Sorsoli and Parks, 1964). In addition, dilution experiments by Parks (1958) have established that AM is preferentially used as the source for the methyl group in this reaction. In this study, methionine was

used whenever possible because of the difficulty and expense of preparing highly labeled AM.

Table 1

Requirements for cell-free sterol synthesis with methionine-methyl-C^{14} as substrate

Reaction	counts/minute
zero control	687
complete	105,583
-ATP	1,141
-G-6-P	106,779
-ascorbate	91,610
-acetate	91,968
-$MnCl_2$	28,885
-K_2CO_3	91,891

Complete control: ATP 60 μm, glucose-6-phosphate 20 μm, potassium ascorbate 20 μm, potassium acetate 120 μm, $MnCl_2$ 4 μm, methionine-methyl-C^{14} 0.2 μm (1.0 μc), 1.5 ml enzyme (54 mg protein).

Table 2

Requirements for cell-free sterol synthesis with AM-methyl-C^{14} as substrate

Reaction	counts/minute
zero control	113
complete	1,309
-ATP	1,765
-G-6-P	1,287
-ascorbate	1,185
-acetate	1,249
-$MnCl_2$	1,232
-K_2CO_3	1,052

Conditions the same as table 1, except S-adenosyl-methionine-methyl-C^{14} 12,334 counts/minute added as the radioactive substrate.

The cofactor requirements when either methionine or AM is used as substrate are similar in most respects. In both cases ascorbate, $MnCl_2$, K_2CO_3 and acetate enhance incorporation. ATP is necessary for incorporation when methionine is used as substrate and inhibits incorporation when AM is used as substrate. In the first case the ATP requirement is anticipated since it is required for the synthesis of AM from methionine; in the second case the inhibition is probably due to the dilution of added radioactive AM by endogenously synthesized AM. The requirement for glucose-6-phosphate has been found to be variable depending on the age of the enzyme preparation; older preparations showing a greater requirement than fresh preparations.

With methionine as substrate the principal requirements for incorporation of radioactivity are $MnCl_2$ and ATP, whereas when AM is the substrate, the principal requirement is K_2CO_3. Additional experiments (table 3) have established that K_2CO_3 is also required for incorporation when methionine is the substrate.

Table 3

Effect of carbonate and Mn^{++} on cell-free sterol synthesis with methionine-methyl-C^{14} as substrate

Reaction	counts/minute
zero control	400
complete	74,577
$-K_2CO_3$	14,225
$-MnCl_2$	41,288

Conditions same as table 1 except cysteine 20 μm was used in place of potassium ascorbate.

It is evident from these data that $MnCl_2$ and ATP are required for AM synthesis and that only K_2CO_3 is required for the transmethylation reaction. Table 4 shows the carbonate effect on incorporation from AM-C^{14} more clearly.

Table 4

Effect of carbonate omission on cell-free sterol synthesis with AM-methyl-C^{14} as substrate

Reaction	counts/minute
zero control	127
complete	1,636
G-6-P	1,628
-ascorbate	1,375
-acetate	1,562
-$MnCl_2$	1,561
-K_2CO_3	734

Conditions the same as table 1, except S-adenosylmethionine-methyl-C^{14} 12,334 counts/minute added as the radioactive substrate; ATP was omitted from reaction mixture. (25 mg protein)

It is difficult to assign exact functions to the requirements for acetate and ascorbate in this reaction except that perhaps the former serves to enhance de novo sterol synthesis and the latter maintains the enzyme in a reduced condition (i.e. sulfhydryl groups).

The carbonate effect could be explained if it is incorporated into the non-saponifiable fraction, perhaps in the formation of an intermediate. However, when this experiment was performed (table 5), it was found that an insufficient amount of carbonate was incorporated

to substantiate this hypothesis. The methionine control was added to insure that the enzyme preparation was active.

Table 5

Incorporation of $Na_2C^{14}O_2$ into sterol by cell-free yeast homogenate

Reaction	counts/minute
zero control (C^{14}-methionine)	890
complete control (C^{14}-methionine)	36,056
zero control ($Na_2C^{14}O_3$)	540
complete control ($Na_2C^{14}O_3$)	1,260

Conditions the same as table 1, except where $Na_2C^{14}O_3$, 0.486 m (1.0 c) was added, no potassium carbonate was added; (30 mg protein) Nonlabeled Na_2CO_3 was added to methionine reactions; nonlabeled methionine was added to Na_2CO_3 reactions.

There has recently been a great deal of interest in the control of cellular systems by specific metabolites and small molecular weight compounds. These do not directly interact with the substrate or the products of reactions but alter in some manner the conformational state of the enzyme protein. The effect has been described as an allosteric transition (Monod, Changeux and Jacob, 1963). It has been possible in the case of l-threonine-deaminase (Changeux, 1961) and phosphoribosyl pyrophosphate-ATP-pyrophosphorylase (Martin, 1963) to destroy or "desensitize' the allosteric site of the protein by heat treatment and $HgCl_2$ treatment respectively.

The carbonate effect observed in the transmethylation reaction in ergosterol synthesis appeared to fit into a role as an allosteric effector. Because of the relatively crude enzyme preparation

employed in this study and since the sterol substrate of the reaction is at present unknown, the only method of identifying the carbonate effect with the allosteric phenomenon was to attempt desensitization of the preparation to carbonate. A number of experiments were conducted employing heating for various times, heavy metal treatment, and storage at low temperature. However, as exemplified by the results shown in figure 1, in every case both the ability of the enzyme preparation to incorporate radioactivity into the non-saponifiable material and its sensitivity to carbonate were destroyed at the same rate. Therefore at present it is not possible to explain the carbonate effect observed in this system as one of an allosteric nature.

In order to substantiate the previously observed transfer of the methyl group of AM (Parks, 1958) and methionine (Alexander, Gold, and Schwenk, 1957; Alexander and Schwenk, 1957) to the side chain of ergosterol, chromatographic separation of the sterols isolated from the cell-free system developed here were carried out. Figure 2 shows the elution pattern obtained from the sterols isolated from a cell-free reaction employing methionine-methyl-C^{14} as the radioactive substrate. Contrary to previous reports, the radioactivity is not found in ergosterol but in two other sterols, one eluting before ergosterol and the major fraction eluting after ergosterol on chromatographic columns.

Figure 3 shows the elution pattern of sterols isolated from whole cells after a 15 minute incubation with methionine-methyl-C^{14}. In this experiment three sterols are found to contain radioactivity-ergosterol and the two sterols isolated from the cell-free reaction.

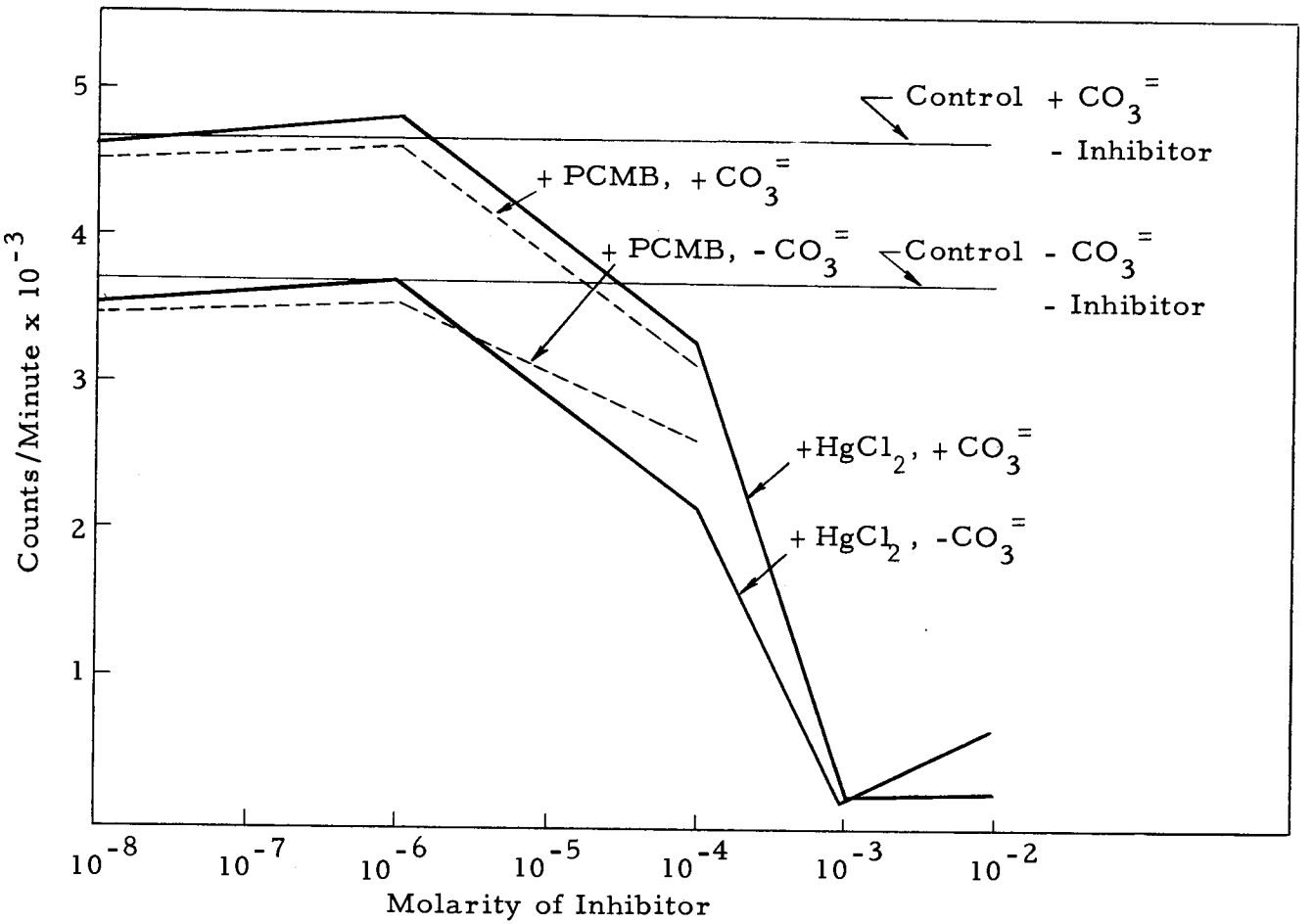


Figure 1. Desensitization of cell-free reaction to the effect of carbonate.

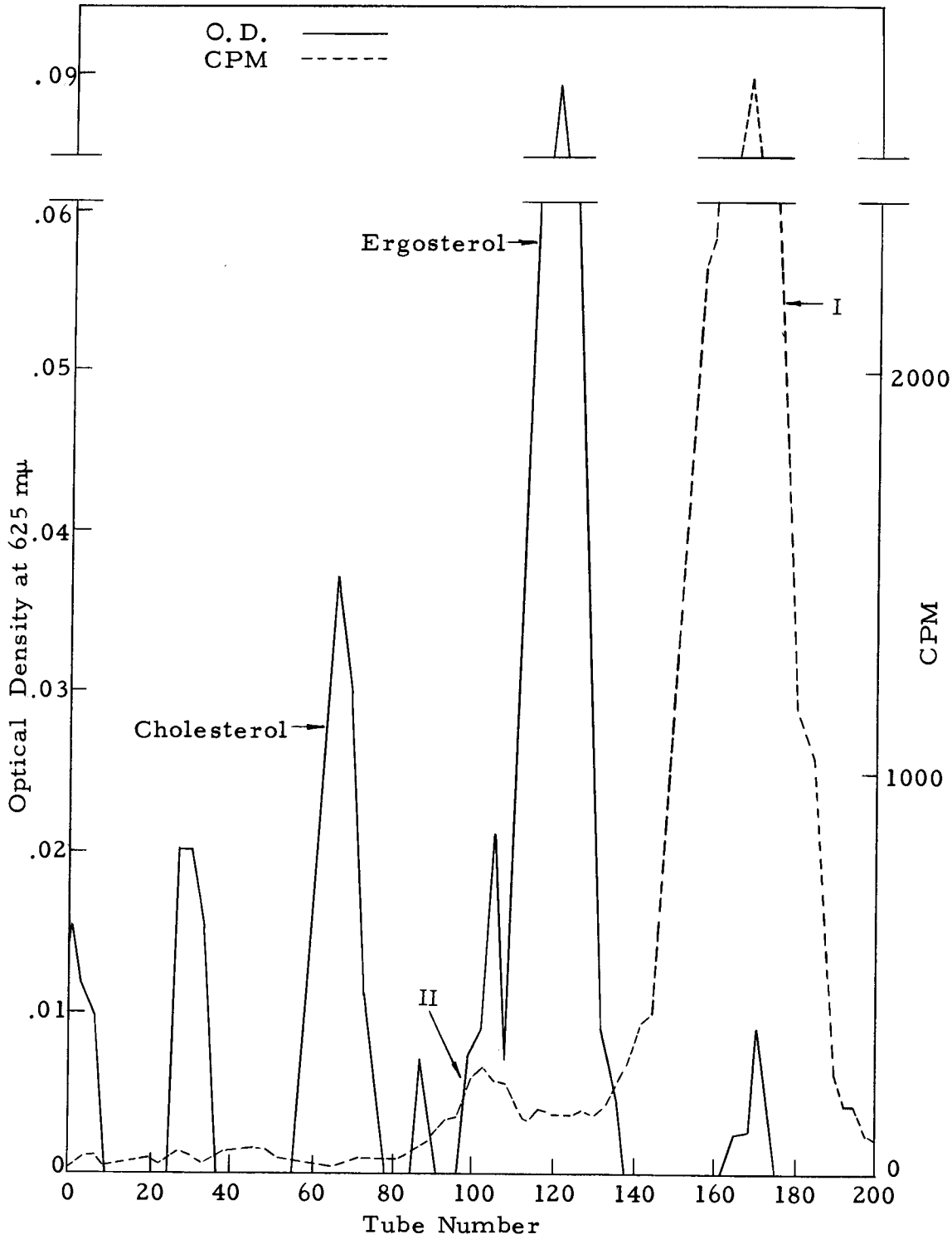


Figure 2. Separation of sterols isolated from cell-free reaction mixture, incubated with methionine-methyl-C^{14}.

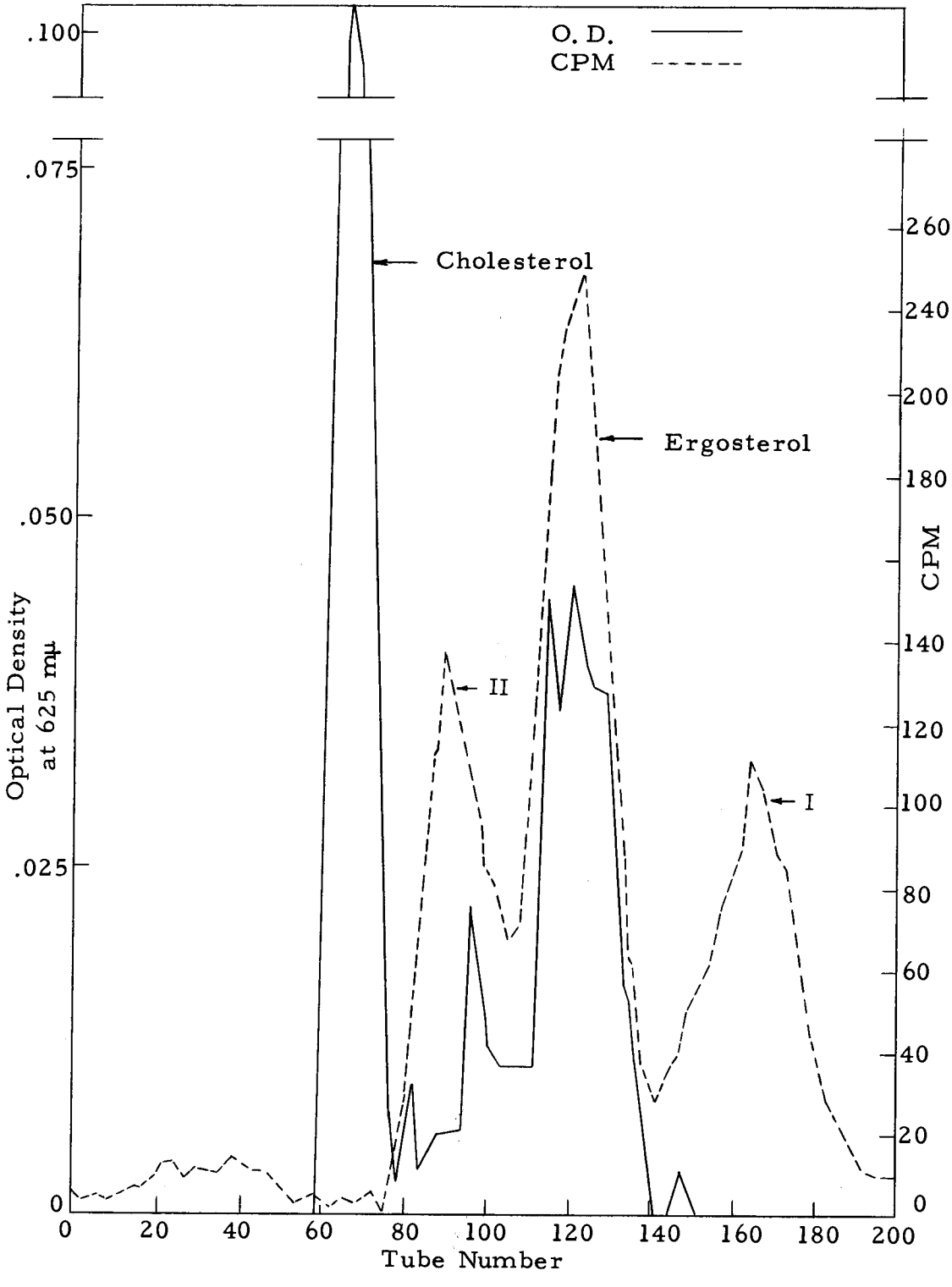


Figure 3. Separation of sterols isolated from whole cells after 15 minutes incubation with methionine-methyl -C^{14}.

Figure 4 and 5 are the elution patterns of the sterols found after 5 and 15 hour incubations of cells from the same experiment. The relative amounts of radioactivity in the three sterols from 15 minutes to 15 hours indicates that there is a rapid turnover of sterol in the cells and that components I and II decrease in amount as ergosterol increases in amount.

Sterol component I was subsequently isolated and incubated with whole cells for 5 hours. The sterols were isolated and chromatographed to determine if component I is converted to ergosterol. Figure 6 shows the results of this experiment. Although a large amount of radioactivity was taken up by the whole cells only a small amount was converted to ergosterol. That this is however a significant amount is shown in table 6.

Table 6

Conversion of component I-C^{14} to ergosterol by whole cells

Reaction	counts/minute
component I added to whole cells	18,000
recovered from whole cells	4,520
separated on thin layer plate	4,520
recovered from plate:	
as ergosterol	1,200
as component I	3,240

The sterols were isolated from a cell-free reaction mixture and separated on a thin layer chromatogram. Component I was reisolated from the plate and incubated with whole cells. Thin layer chromatography of the sterols followed by recovery and determination of

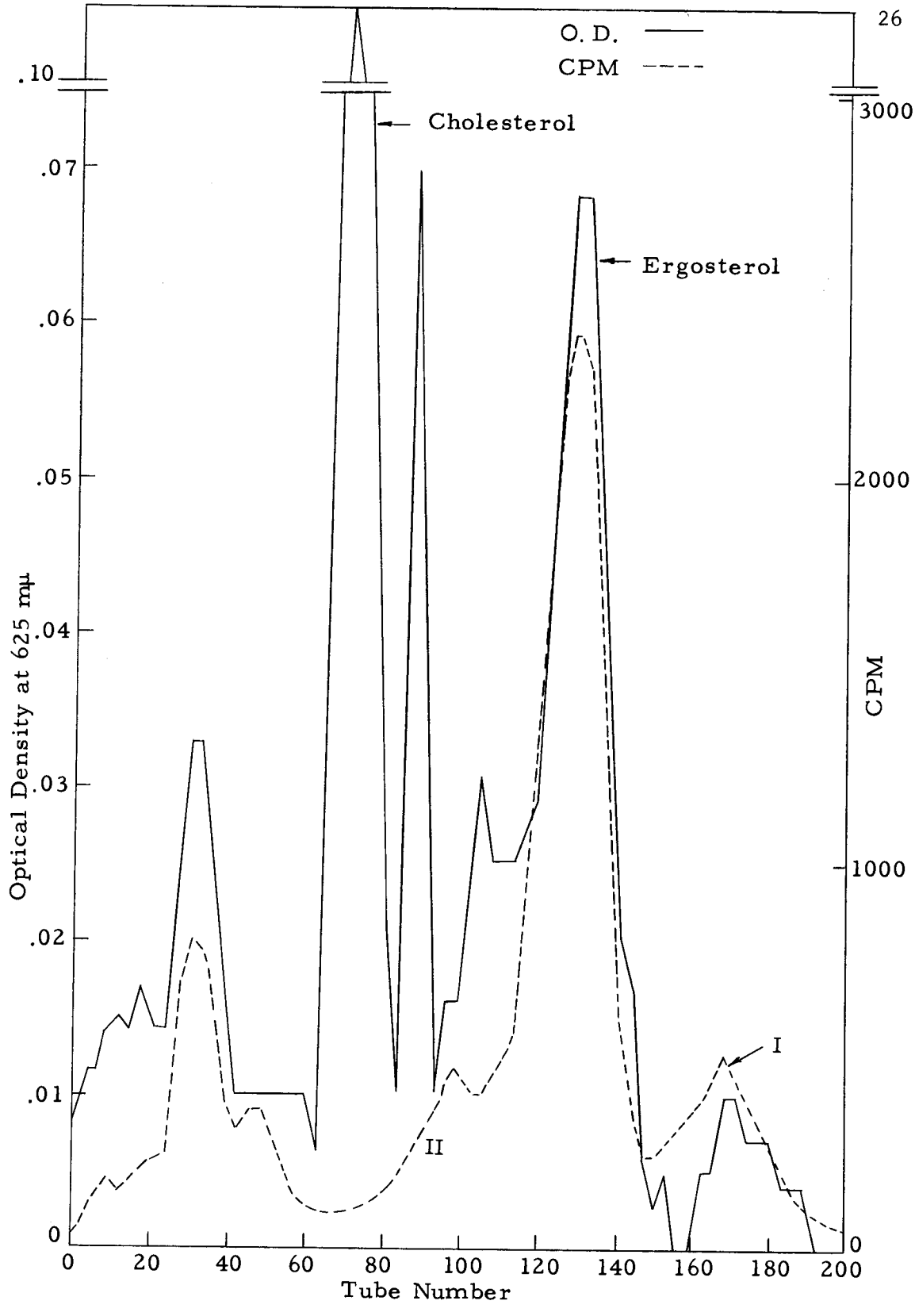


Figure 4. Separation of sterols isolated from whole cells after 5 hours incubation with methionine-methyl -C^{14}.

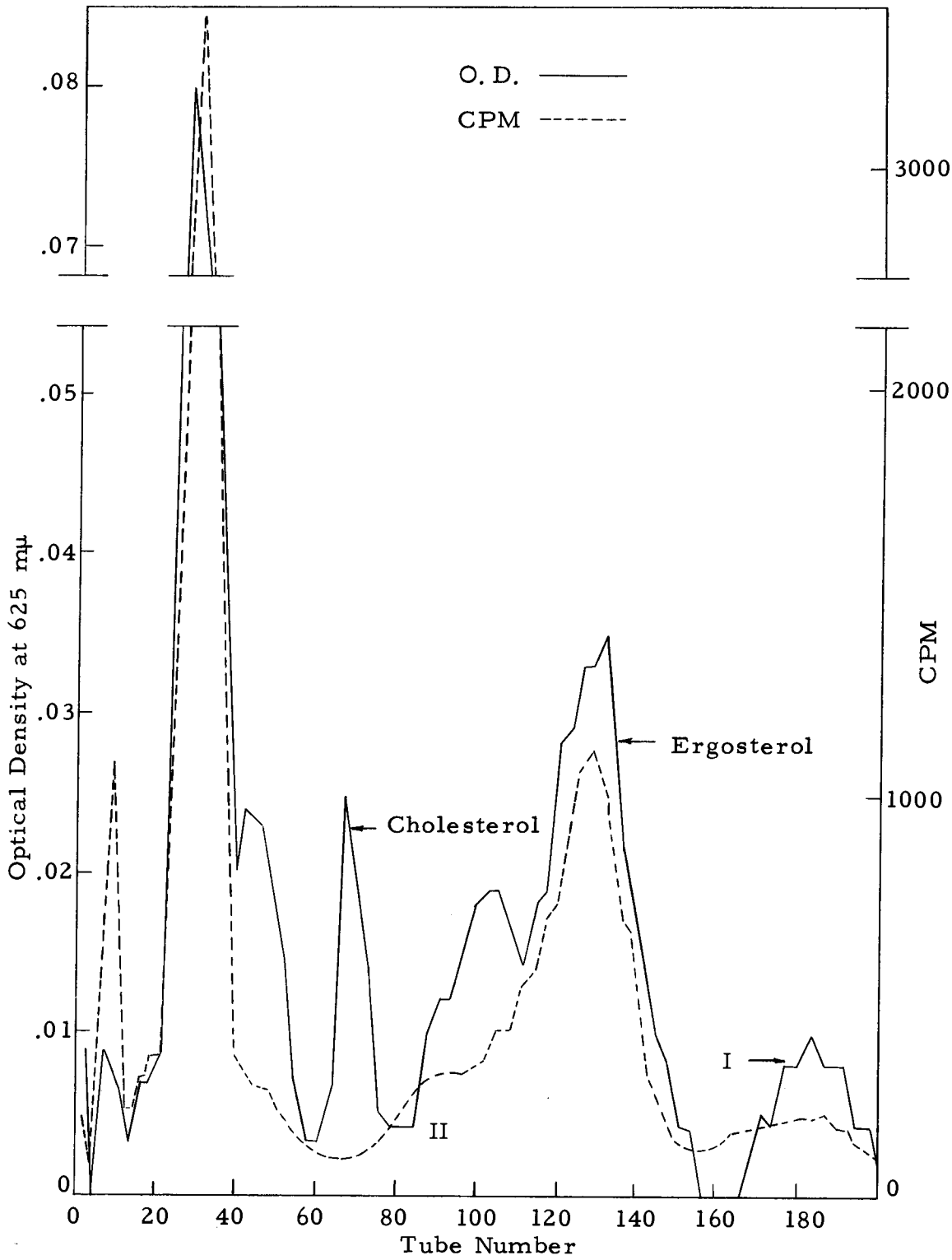


Figure 5. Separation of sterol isolated from whole cells after 15 hours incubation with methionine-methyl-C^{14}.

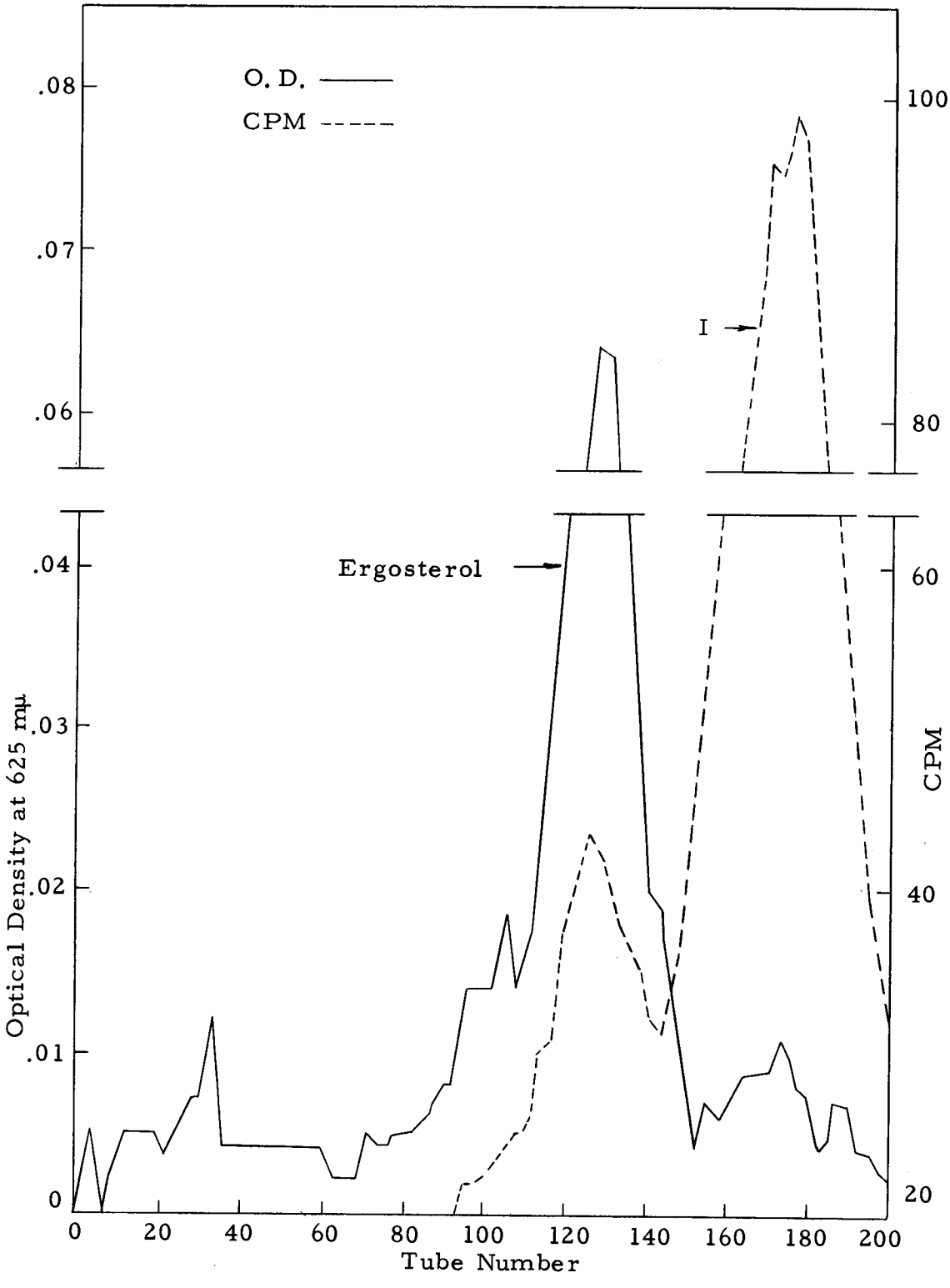


Figure 6. Separation of sterols isolated from whole cells after 5 hour incubation with component I-C^{14}.

radioactivity was performed.

Of the 18,000 counts added to the whole cell incubation, 4,520 counts or 25% was recovered, and of the radioactivity recovered from the thin layer plate, 27% was found in the ergosterol. There is little doubt then that this sterol is a precursor of ergosterol. There has not been sufficient amounts of component II isolated to determine if it also is a precursor of ergosterol. However, from time course experiments with whole cells it is probable that both of these sterols are precursors of ergosterol.

DISCUSSION

A cell-free system has been developed from yeast which is capable of incorporating the methyl group of S-adenosylmethionine or methionine into the non-saponifiable material. This system requires for maximal activity the presence of K_2CO_3 when AM is the methyl donor and ATP, $MnCl_2$ and K_2CO_3 when methionine is the methyl donor. Ascorbate, acetate and glucose-6-phosphate enhance the activity about equally with both substrates. It is reasonable to assume that the carbonate requirement is concerned then with the transmethylation reaction proper.

An attempt was made to equate the carbonate effect found in this system with the allosteric transition phenomenon demonstrated in other metabolic pathways (Monod, Changeux and Jacob, 1963). However, using the methods previously employed with success in other systems to demonstrate an allosteric site on the protein (Changeux, 1961; Martin, 1962), it has not been possible to find any evidence that carbonate ion plays the role of an allosteric effector in this transmethylation reaction. Although desensitization experiments were unsuccessful in producing a specific inactivation of the response of the system to carbonate, two observations still leave the possible allosteric action of this compound as an attractive explanation for its role in this transmethylation. These are (1) the specific enhancing effect of carbonate on the incorporation of the methyl group of AM into sterol and (2) the low incorporation of radioactive carbonate into sterol.

Since the allosteric transition phenomenon has been implied to range in effect from alteration of the conformation of a single protein to affecting the aggregation of inactive protein subunits into an active complex, desensitization of a particular system to its allosteric effector is probably a highly specific process. This would explain why specific treatments successful in other systems did not work in the transmethylation reaction studied here. It will therefore be necessary to examine additional methods of desensitization and to develop a more highly purified enzyme system before an allosteric interaction of carbonate can be confirmed or discarded.

It was mentioned earlier that the low incorporation of carbonate into sterol probably would rule out any involvment of carbonate in the biosynthesis of an intermediate of sterol synthesis. This explanation does not however exclude the possibility that carbonate may participate in the formation of a transient intermediate which is rapidly turned over and thus would not have been detected in large amounts by an instantaneous assay for incorporation of radioactive carbonate. The most probable carbonate intermediate of sterol synthesis one could predict at this late stage in the pathway would be a carbonic acid ester at the 3-β-hydroxyl group. Although carbonic acid esters are well known in organic chemistry (i.e. diethyl carbonate), the role of a mono or dialkyl carbonate in the formation of metabolic intermediates directly synthesized from carbonic acid have not been demonstrated. However, phosphoric acid esters are common as transient intermediates in biosynthetic reactions and recently

sulfuric acid esters of cholesterol have been found in bovine adrenals (Drayer et al., 1964) and in human blood and gallstones (Drayer et al., 1965). Other derivatives of sterols have been found in both mammalian and plant systems. Fatty acid esters of sterols have long been observed in mammalian and yeast systems and recently a glycoside derivative of sitosterol has been isolated from plants (Lepage, 1964). It has not been established what role these various derivatives play in either the biosynthesis or metabolism of the particular sterols. The important fact at this point is that they have been demonstrated to occur naturally.

Evidence of another type which indicates that sterols probably are not synthesized as the free compounds but as some type of derivative, stems from studies of incorporation of radioactive precursor sterols. Clayton and Bloch (1956), showed that after incubation of C^{14}-lanosterol with a rat-liver homogenate only 2.6 to 19% of the radioactivity was converted to cholesterol. This type of result is common in most studies of incorporation of free sterol, that is, conversion of the free sterol to end product is very low. As shown in table 6 of this investigation, in which a radioactive precursor of ergosterol was incubated with whole cells, a relatively small amount of the precursor sterol was converted to ergosterol. Since 98% of the sterol taken up by the cells can be accounted for and 73% of this material was recovered as the starting material, it can be predicted that this low conversion is due to the inability of the cells to convert free sterol to an intermediate.

Evidence has been presented here which indicates that the transmethylation reaction probably occurs at least two steps prior to the synthesis of ergosterol. This is clear from the chromatographic separation of cell-free and whole cell sterols which contain the methyl group from AM. This confirms the prediction made by Danielsson and Bloch (1957) that the C-24 methyl group is added at a late stage in ergosterol synthesis. This was inferred from the fact that formate-C^{14} did not contribute any label to squalene, lanosterol or zymosterol, but only to ergosterol.

Experiments have been carried out and are continuing in an attempt to isolate enough of the precursors of ergosterol for determination of their structural formulas. Some inferences on structure may be made at this point however, on the basis of previous work by others and chromatographic work done in this investigation.

It can be seen from the chromatographic separation that ergosterol is not labeled in the cell-free system, but is labeled by the whole cell system. Also, the two sterol precursors of ergosterol are labeled in both the cell-free and whole cell systems. Therefore any work performed on the cell-free system products would not be a measurement of the synthesis of ergosterol but of the synthesis of the two precursors, and any measurement of the sterols isolated from whole cells would measure not only ergosterol but also the two precursors.

Alexander, Gold and Schwenk (1957) degraded, by ozonolysis, the non-saponifiable fraction from cell-free yeast homogenates which had been incubated with methionine-methyl-C^{14}. Ozonolysis breaks the C-22

double bond of the side chain yielding α-β-dimethylbutyraldehyde which contains C-23 to C-28 of the supplied sterol. Further degradation of this aldehyde allows isolation of each atom of the fragment and consequently each carbon atom can be assayed for radioactivity. They found that carbon 28 contained 20-30 times the radioactivity of any carbon atom of the molecule when the source of radioactivity was methionine-methyl-C^{14} in their cell-free biosynthetic mixture. However, as seen from this study, the cell-free system from yeast does not yield labeled ergosterol from methionine-methyl-C^{14}, therefore the assay for radioactivity in the side chain was that of the precursors and not that of ergosterol.

Danielsson and Bloch (1957) and Hanahan and Wakil (1953), performed similar experiments in degradation of yeast sterols but in these cases the sterols were isolated from whole cells. They found that C-28 contained many times the radioactivity of any other carbon atom of the molecule, and in the experiments of Danielsson and Bloch, all of the radioactivity from formate-C^{14} was found in C-28. It can therefore be predicted that the two precursors of ergosterol isolated in this investigation have a double bond at C-22, as does ergosterol.

The structural differences between the precursors and ergosterol therefore must reside in the number and position of the double bonds in the ring system. Silicic acid column chromatographic evidence obtained from the techniques of Klein and Szczepanik (1962) show that precursor I has an elution behavior similar to that of zymosterol which has double bonds at C-8 and C-24. Thin layer chromatographic behavior of this precursor according to the procedure of Avigan,

Goodman and Steinberg (1964) demonstrates that this sterol behaves as sterols which have a single double bond in the ring system, like cholesterol. Sterols with different substituents in the side chain are not separated from each other by this method (i.e. cholesterol and desmosterol).

Ultraviolet absorption of sterols depends on the presence of a conjugated diene in the ring system of the molecule. Fieser (1937), noted that cyclic dienes fall into two groups, heteroannular dienes and homoannular dienes. Heteroannular dienes such as $\Delta^{3,5}$ -cholestadiene have maxima in the region 220 to 250 mμ and extinction coefficients of 14,000 to 28,000 while homoannular dienes such as ergosterol have maxima in the region 260 to 285 mμ and extinction coefficients of 5,000 to 15,000. Ultraviolet absorption curves prepared for the two ergosterol precursors isolated from yeast show no absorption maxima from 220 to 300 mμ.

At the present then we can predict that the two precursors of ergosterol isolated in this study have double bonds at C-22 and have only one double bond in the ring system.

It appears from the evidence presented here concerning the sequence of synthesis and structure of the sterols which are the progenitors of ergosterol that the same general pattern occurs in ergosterol synthesis as occurs in cholesterol synthesis. The essential features of this pattern are that the structure of the side chain is established early in synthesis and is followed by rearrangements of the position and number of the double bonds in the ring system.

SUMMARY

The role of S-adenosylmethionine in the biosynthesis of ergosterol has been investigated. It was found that cell-free yeast homogenates require K_2CO_3, acetate, $MnCl_2$, ascorbate and glucose-6-phosphate for maximal activity in the transfer of the methyl group from AM to the side chain of sterols. The possibility that K_2CO_3 functions as an allosteric effector for this enzyme was tested. With the methods used for desensitization of the enzyme to carbonate it was not possible to demonstrate this relationship.

Data derived from chromatographic separation of the sterols synthesized in both the cell-free and whole cell systems indicate that the C-24 methyl group is transferred to a preformed sterol nucleus at a late stage of synthesis. It was demonstrated that there are two precursors of ergosterol which contain the methyl group. Therefore the transmethylation occurs at least two steps prior to the formation of ergosterol.

Evidence is presented which indicates that the precursors have the same side chain unsaturation as does ergosterol and differ in the number and position of the double bonds in the ring system.

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