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The regulation of ergosterol biosynthesis in yeast has been studied. The system has been examined for the presence of an end-product inhibition. Both anaerobically and aerobically grown cells have been examined for this effect. A feedback inhibition of sterol synthesis has been shown, but it appears to be a secondary effect. The regulation is intimately involved with the carbohydrate metabolism of the aerobic cell. Other involvements may exist in anaerobic cells. A type of catabolite repression of ergosterol biosynthesis has been hypothesized.

An inhibitor of sterol synthesis which is cationic in nature has been found in yeast extract. The inhibitory effect is directed specifically at transmethylation, and may cause the accumulation of precursors to transmethylation in ergosterol synthesis.

A procedure has been developed for extracting sterols from

cell-free extracts of yeast. The method gives larger and more consistent yields of sterol than the conventional methods of extraction.

Aspects of Ergosterol Biosynthesis in *Saccharomyces cerevisiae*

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To Britt

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INTRODUCTION

Ergosterol comprises on the average two percent of the dry weight of the yeast cell. Despite the large amount of sterol in that organism, very little is known of the role of ergosterol in the metabolism of the cell.

Most of the research on ergosterol has been directed towards elucidating the biosynthetic pathway for its synthesis. The conversion of acetate to lanosterol has been well documented, and is the same for both cholesterol and ergosterol biosynthesis. The terminal steps of ergosterol synthesis have yet to be established.

Of the many enzymatic steps leading to the formation of cholesterol from acetate, three have now been shown to be sensitive to an allosteric inhibition by cholesterol. Since the biosynthesis of ergosterol so closely resembles that of cholesterol, it is assumed that yeast exert some control over ergosterol synthesis. The ergosterol synthesizing system has therefore been studied to determine if there exists some form of end-product regulation of yeast sterol formation.

The work with cholesterol has also shown that the control of

its synthesis is dependent to a certain extent upon the organ in which synthesis takes place. In yeast, relationships have been previously established between ergosterol and the respiratory competency of the cell, and between ergosterol and the levels of some dehydrogenase enzymes in the organism. These relationships have been studied for their possible involvement in the regulation of ergosterol biosynthesis.

The development of some new methodology has been necessary in order to obtain accurate answers to the questions asked in this study. The results obtained using these new procedures have been formulated into some theories concerning the regulation of ergosterol biosynthesis and the physiological function of ergosterol in the cell.

LITERATURE REVIEW

Compounds belonging to the classification called "steroids" can all be considered to be derivatives of perhydrocyclopentanophenanthrene, a completely saturated ring system consisting of three cyclohexane molecules in the phenanthrene configuration with a terminal cyclopentane group. Steroids containing eight to ten carbon atoms in a side chain at position 17, and an alcoholic hydroxy group at position three are classified sterols. Ergosterol is a sterol with two double bonds in the B ring at positions five and seven, and another double bond in the side chain at position 22. The side chain of ergosterol contains nine carbon atoms, the 28th carbon atom being bonded to carbon 24 of the side chain.

Ergosterol was first isolated by Charles Tanret in 1889, who gave the name ergosterol to the substance he had isolated from ergot of rye. The physical constants of melting point and specific rotation which he determined have been verified and are still considered reliable.

Smedley-MacLean and Thomas (1920) were the first to isolate ergosterol from yeast in large quantities. Since that time yeast has been a major source of ergosterol, comprising approximately two percent of the dry weight of the cell.

Research pertaining to ergosterol remained very limited,

however, compared to the large amount being done with cholesterol, until the search for the antirachitic provitamin began to center on ergosterol (Rosenheim and Webster, 1927). With the discovery that ergosterol, or a sterol very similar to ergosterol, was provitamin D, elucidation of the structure of ergosterol began. It was not until six years later that the complete structure of the molecule was known, when Chuang (1933) confirmed the structure of the side chain of ergosterol.

Establishment of the biosynthetic pathway for ergosterol has followed two courses. The first, the synthesis of the steroid ring system from carbohydrate precursors, parallels that found for the biosynthesis of cholesterol from acetate. Clayton and Bloch (1956a, b) first demonstrated that acetate can serve as the major carbon source for cholesterol and ergosterol. Studying the distribution of C^{14} label in ergosterol biosynthesized from acetate-1-C^{14}, Dauben, Hutton and Boswell (1959) concluded that ergosterol is synthesized via squalene, and that the squalene hypothesis, which predicted that all sterols are synthesized via squalene, was valid for ergosterol. Cornforth (1959) showed that many of the enzymes involved in the formation of squalene from acetate were the same for both the cholesterol and ergosterol systems. These findings established that the biosynthetic pathways through the cyclization of squalene are the same for both cholesterol and ergosterol.

The second line of investigation concerned the establishment of the sequence of steps involved in the conversion of lanosterol to ergosterol: demethylation, formation and positioning of the two double bonds in the B ring, unsaturation of the side chain in position 22, and addition of the 28th carbon to carbon 24 of the side chain. In 1953 Hanahan and Wakil biosynthesized ergosterol from carboxyl labeled acetate. Analysis of the side chain of the labeled ergosterol showed that virtually all of the label was contained in carbon atoms 23 and 25, and that none was in the C-24 methyl group. This indicated a source for the methyl group other than acetate. That the 28 carbon of ergosterol could come from a source other than acetate was first demonstrated by Danielson and Bloch (1957). They analyzed ergosterol derived from resting cells of yeast incubated 24 hours in glucose and formate-C^{14}. All of the C^{14} taken up by the yeast went into ergosterol, and ozonolytic cleavage of the side chain showed that virtually all of the label was contained in carbon 28. Alexander, Gold and Schwenk (1957) incubated cell-free extracts of yeast with C^{14} labeled bicarbonate, formate, propionate and methionine (methyl-C^{14}) and found that methionine was by far the most efficient in contributing the 28 carbon of ergosterol. Parks (1958) demonstrated by competition experiments that the actual methyl donor in ergosterol biosynthesis is the activated form of methionine, S-adenosylmethionine.

This work established the origin of carbon 28, but it did not

indicate at what stage in the formation of the ergosterol molecule the transmethylation takes place. It also did not help to establish the sequence of events occurring between lanosterol, the product of the cyclization of squalene, and ergosterol. While the conversion of lanosterol to cholesterol has been well documented, the terminal steps in ergosterol biosynthesis have been only partially determined. Kodicek and Ashby (1957) first demonstrated the direction of synthesis. Using anaerobically grown cells, they aerated the resulting sterol deficient yeast for 24 hours with acetate-1-C^{14}, and analyzed for sterols at intervals during the aeration period. Squalene decreased from 66% to 21% of the unsaponifiable matter after two hours, and was only eight percent of that fraction after 24 hours. Lanostadienol increased from 0.5% to 35% of the unsaponifiable fraction in four hours, then decreased to 12% after 24 hours. Ergosterol increased slowly, reaching a maximum of 35% at 24 hours. Zymosterol, the second most prevalent sterol in yeast, ran parallel to ergosterol. The results suggest a pathway from squalene to lanostadienol to ergosterol. Alexander and Schwenk (1958) proved that although zymosterol can be converted to cholesterol, it is not converted to ergosterol. Labeled rat lipids, squalene, and lanosterol gave rise to labeled ergosterol in cell-free yeast homogenates, but zymosterol did not.

That the final step in ergosterol biosynthesis was not

transmethylation was shown by Turner and Parks (1965). They demonstrated the existence of two methylated intermediates in yeast cell-free ergosterol synthesizing systems and in whole cells of yeast. One of these methylated compounds, when isolated from cell-free reactions and fed to whole cells, was partially converted to ergosterol. Not enough of the second intermediate could be isolated to perform similar experiments, but the relative time of its appearance was suggestive of it too being an intermediate.

Katsuki and Bloch (1967) proposed that the structure for component 1 of Turner and Parks (1965) was 5, 7, 22, 24 (28)-ergostatetraene-3β-ol. This formula had previously been suggested for a sterol isolated from yeast (Breivik, Owades and Light, 1954). Katsuki and Bloch (1967) also found one other methylated component. Both components which they analyzed had the carbon skeleton of ergostane, leading them to predict that methylation occurs following the demethylation of lanosterol.

Other groups (Akhtar, Parvez, and Hunt, 1966, and Barton, Harrison, and Moss, 1966) have given evidence that labeled 24-methylenedihydrolanosterol is converted into ergosterol in whole cells of yeast. From this they have suggested that methylation on carbon 24 occurs at the stage of lanosterol. Akhtar, Hunt and Parvez (1966) studied the conversion of 24-H^3-lanosterol and 26, 27-C^{14}-lanosterol to ergosterol. They showed that both labels were

incorporated at the same rate into ergosterol, and that the H^3 atom had migrated from C-24 and was not located at C-23. From this they proposed a mechanism for the methylation of lanosterol involving a hydride ion shift from C-24 to C-25.

The proposal for the methylation of lanosterol has also been challenged indirectly by Ponsinent and Ourisson (1965), who made a comprehensive survey of the methyl sterols of yeast (those related to lanosterol, having two extra methyl groups at position 4 and one at position 14). They found no trace of any 24-methylene derivatives of this class of sterols.

As mentioned earlier, in a wild type strain of Saccharomyces cerevisiae approximately two percent of the dry weight is composed of sterol, most of it being ergosterol. A compound occurring in such abundance would seem to have some obvious physiological function. Several observations have been made which suggest possible functions for this sterol, the most interesting being the relationship between ergosterol and the aerobic metabolism of the cell. As early as 1940, Maguigan and Walker noted that aerobic yeast cells contained large amounts of sterol, whereas anaerobic cells did not. From this and other work they concluded that ergosterol was intimately involved with aerobiosis.

That oxygen is necessary for the synthesis of ergosterol can be concluded from the work with cholesterol. It has been shown

(Tchen and Bloch, 1957) that molecular oxygen is necessary for the cyclization of squalene into lanosterol in rat liver homogenates. In the presence of O_2^{18}, these homogenates formed lanosterol containing O^{18}. Also, no lanosterol was formed under anaerobic conditions. At the same time Olson, et al. (1957) showed that molecular oxygen is necessary for the demethylation of lanosterol. From methyl labeled acetate they biologically synthesized lanosterol. The methyl groups of the lanosterol thus synthesized were labeled. In the biological conversion of that compound to cholesterol the methyl groups were released as $C^{14}O_2$. This type of experiment has not been done in yeast, but Klein (1955) has shown that during anaerobic growth yeast synthesize squalene, but not sterols. Upon subsequent aeration in glucose and phosphate buffer, anaerobically grown yeast rapidly begin synthesis of sterols. Klein has further shown that virtually all of the glucose-C^{14} label given to yeast during aeration in a resting suspension is converted to sterol. From this he concluded that the squalene pool formed during anaerobic growth is not used for sterol synthesis in resting cells as long as there is glucose present.

An interesting paradox to the requirement for oxygen for sterol synthesis has been shown by Andreason and Stier (1953, 1954). They demonstrated that yeast will not grow under strictly anaerobic conditions unless the medium is supplemented with ergosterol and an

unsaturated fatty acid.

Parks and Starr (1963) showed a relationship between ergosterol and respiratory competency in yeast. Using metabolic inhibitors and metal ions known to induce respiratory deficient petite strains, it was found in every case that these substances inhibited sterol synthesis in resting cells. Furthermore, it was noted that added ergosterol reduced the numbers of petites which these substances could produce.

Other evidence which supports this type of result has been presented by Morpurgo, et al. (1964). Looking at the morphological features of the yeast cell, they found that cells grown anaerobically in the presence of ergosterol contained all of the structural features of aerobic cells, including well defined mitochondria. In cells grown anaerobically without ergosterol, growth stopped after 5 to 7 generations and the cells had little internal structure, the mitochondria being absent. The life span of these cells was inversely proportional to the number of cell divisions under anaerobiosis, and death was due to lysis. From these results, they predicted a membrane involvement for ergosterol, the lack of ergosterol causing improper membrane formation.

The involvement of ergosterol in respiratory metabolism has been approached from another angle by work which began with Tauson (1948). In adding crystalline ergosterol to agar mash cultures of

Saccharomyces carlsbergensis and Endomyces magnussi, he found that concurrently with an increase in dry weight and fermentative activity, there was a decrease in the rates of dehydrogenation of glucose and succinate. From this he concluded that ergosterol shifts the oxidation-reduction potential towards reduction by inhibiting dehydrogenases. Matkovics (1958) also observed this effect in Penicillium chrysogenum, noting that ergosterol added in a concentration of 0.16% markedly inhibited dehydrogenase formation.

A specific involvement of ergosterol with an enzyme has been shown by Deborin, et al. (1960). They have shown that ergosterol binds in minute quantities at low pH to phosphoglyceraldehyde dehydrogenase. The binding causes changes in the viscosity, optical rotation, and molecular weight of the enzyme. They predicted that the result of the binding of the ergosterol to the enzyme is the prevention of the transfer of hydrogens to NAD^+.

A corollary to the work implicating the involvement of ergosterol with dehydrogenases has been shown. Thioglycolic acid, which lowers the oxidation-reduction potential of a culture, when added to a culture of yeast nearly doubles the yield of ergosterol (Kramli and Lantos, 1956).

The regulation of ergosterol biosynthesis is an important factor in its synthesis by the cell. This aspect has not been studied in yeast. However, control over cholesterol synthesis in

mammalian systems has been studied extensively. As is the case with the biosynthetic schemes, analogous regulatory phenomena may exist between cholesterol and ergosterol. In hepatic cholesterol biosynthesis a negative feedback inhibition was first evidenced by the depression of the rate of synthesis of cholesterol following cholesterol feeding (Tomkins, Sheppard and Chaikoff, 1953). Indirect evidence was later given by Siperstein and Guest (1960) that the site of control was the enzyme mediating the reduction of β-hydroxy-β-methylglutaryl coenzyme A (HMG-Coa) to mevalonic acid (MVA). Because the inhibition was immediate, they predicted a feedback inhibition rather than a repression. Direct evidence has recently supported HMG-CoA reduction as the site of the feedback inhibition. Linn (1967a, b) has isolated the HMG-CoA reductase from rat liver microsomes and shown that the activity of the enzyme is depressed in rats fed cholesterol. Gould and Swyryd (1966) have shown, however, that in addition to the HMG-CoA reductase site, there are two other sites of control. One site is between MVA and farnesyl pyrophosphate, and the other is at the conversion of farnesyl pyrophosphate to squalene. Both are sensitive to feedback inhibition. Because HMG-CoA reduction is the rate limiting step in normal cholesterogenesis from acetate, it is predicted to be the most important site of control. The others can be considered to be effective only under conditions of long term cholesterol feeding.

Feedback inhibition cannot be considered the only means for the regulation of cholesterol synthesis. Ogilvie and Kaplan (1966) have shown that a small molecular weight protein fraction isolated from bile will markedly inhibit cholesterol biosynthesis in rat liver homogenates. This inhibition is much greater for the incorporation of acetic acid than for mevalonic acid. There is no inhibition of fatty acid synthesis, indicating that this may also be an important modulator of cholesterol synthesis. Another possible mode of regulation has been shown by Nightingale, et al. (1967). In homogenates of rat testicular tissue the demethylation of lanosterol is inhibited as much as 81% by several steroid hormones, progesterone and testosterone being most effective.

The knowledge pertaining to the physiological function of ergosterol and the regulation of its biosynthesis is thus very limited. The work done on the cholesterol system can however be used as a guideline for studying the metabolism of ergosterol in yeast.

MATERIALS

Reagent grade dimethylsulfoxide and petroleum ether (b.p. 30-60°C) were purchased from J. T. Baker Chemical Company, Phillipsburg, N.J. Ergosterol was from Sigma Chemical Company, St. Louis, Missouri, and lanosterol from Mann Research Laboratories, New York, N.Y. Zymosterol was a product of Fleischmann Laboratories, New York. Digitonin was from Calbiochem, Los Angeles, California. Sephadex was a product of Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Silica Gel G was from Brinkman Instruments, Inc., Westbury, New York. 2, 5-diphenyloxazole (PPO) and 1, 4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) were purchased from Packard Instrument Company, Downers Grove, Illinois. L-methionine-methyl-C^{14} and sodium acetate-1-C^{14} were from New England Nuclear Corporation, Boston, Mass. All other chemicals used were of reagent grade.

METHODS

Cultures

A wild type strain of Saccaromyces cerevisiae, designated MCC, was used throughout this work. Cultures were maintained at 4°C on agar slants and were transferred monthly. Seed flasks for inoculating large amounts of media were obtained by growing strain MCC in 50 ml of TCA medium at 30°C on an incubator-shaker for 24 hours.

Media

TCA medium for seed flask cultures consisted of 1% glucose, 0.5% yeast extract, and 1% tryptone.

The medium used for the growth of large amounts of cells under anaerobic conditions was designated YMAF. Its composition was 2% glucose, 1% yeast extract, 0.1% ammonium chloride, 1.1% K_2HPO_4 and 1.85% KH_2PO_4. The glucose was autoclaved separately in preparing the medium. For anaerobic growth, 1700 ml of the medium was placed in a 2 liter flask and autoclaved. Immediately following this, 100 ml of sterile glucose was added and the medium allowed to cool before inoculation.

Aeration medium for activating sterol synthesis in anaerobically grown cells was composed of 1% glucose and 0.1 molar KH_2PO_4.

Anaerobic growth of cells

To study the process of sterol formation, the cells were grown according to the anaerobic-aerobic technique of Klein (1955). This process allows the separation of sterol synthesis into two phases, the anaerobic formation of squalene, and the aerobic cylization of squalene and terminal steps of sterol biosynthesis. Anaerobic growth was achieved by inoculating one or more flasks of YMAF medium each with 1.0 ml of cell suspension from a TCA seed flask. The flasks were then placed in a 30°C water bath and incubated for 40 hours. Following incubation the flasks were placed at 4°C overnight to allow the cells to settle to the bottom of the flask so that they could be harvested.

Aeration of anaerobically grown cells

Aeration of the anaerobically grown cells is necessary to activate some of the enzymes responsible for the terminal steps of sterol biosynthesis. The aeration was performed by placing the cells in the aforementioned aeration medium and incubating at 30°C for 2 hours on a reciprocating shaker. Unless stated otherwise, 100 ml of aeration medium was used per 20g of wet cells.

Preparation of cell-free extracts

Two methods were used for the preparation of cell-free extracts. One was the method of Klein (1957). Cells grown as above by anaerobic growth and subsequent aeration were washed two times with cold 0.1 M phosphate buffer (pH 6.5). The wet packed cells remaining after discarding the supernatant from the second wash were spread, with the aid of a rubber policeman, in a thin, even layer on the end of a large pestle which had been previously cooled to -50°C. The pestle was then plunged into powdered dry ice to freeze the cells, then the frozen cells removed from the pestle into a cold mortar. This process was repeated until all of the wet packed cells had been similarly frozen. A small amount of powdered alumina was then added to the mortar, and the frozen cells ground vigorously for 20 min. Approximately 1 ml of phosphate buffer per 20 g of wet packed cells used was mixed into the mixture as it thawed. After thawing, the ground cells were centrifuged at 3020 x g in a Servall RC-2 refrigerated centrifuge for 10 min. to remove the cell debris. The liquid layer resulting from centrifugation was carefully pipetted from the solid layer, leaving the floating lipid layer behind at the same time. This extract was then passed through a column of Sephadex G-50 equilibrated in the cold with 0.1 M phosphate buffer. The resulting desalted crude cell-free extract was used in the cell-free experiments.

In the other method for obtaining cell-free extracts, an Eaton press was used (Eaton, 1962). A thick suspension of the washed cells was made by mixing into them approximately 0.5 to 1.0 ml of phosphate buffer per 20 g of cells. An 8.0 ml aliquot of this suspension was then pipetted into an Eaton press, which had been cooled in dry ice, and the press returned to the dry ice for 20 min. to thoroughly freeze the cells. Then the piston was placed in the cylinder and the press placed under 10,000 pounds pressure until the cells were driven through the press. The broken cells were then thawed, 1.0 ml of cold phosphate buffer per 20 g of original cells added and mixed in, and the mixture centrifuged at 12,100 x g for 10 min. The liquid layer was decanted and treated as described above. The resulting fraction was used for cell-free reactions.

Cell-free reactions

To each cell-free reaction was added 15.0 μm adenosine triphosphate (ATP), 20.0 μm potassium ascorbate, 60.0 μm potassium bicarbonate, 4.0 μm manganese chloride, 1.0 μc of radioactive substrate, and 1.0 ml of the cell-free enzyme. The reactions were then incubated without shaking at 30°C for 5 hours.

Addition of sterols to aqueous mixtures

To add sterols by petroleum ether evaporation, the sterols, dissolved in petroleum ether, were added in the desired amounts to the reaction flasks prior to the addition of any aqueous constituents, then the solvent evaporated under a stream of nitrogen.

Addition of sterols to solutions containing the soluble sterol complexing agent was achieved by adding the sterols in 95% ethanol solution to the complex solution, mixing well, then heating for 10 min. at 100°C in an Arnold sterilizer to remove the ethanol. Control flasks to which no sterol was to be added were treated by adding to them a volume of 95% ethanol equal to that added to the sterol containing flasks.

Extraction of sterols from cell-free reactions

When precipitation of the protein from the cell-free reaction mixtures was desired as a step in extraction, it was achieved by chilling both the cell-free reaction mixture and a 10% solution of trichloroacetic acid (TCA) in an ice bath, then adding 0.3 ml of the TCA solution to each reaction mixture (approximately 40 mg protein per reaction mixture). A final concentration of 2% TCA was obtained.

Two methods of saponification were used for the cell-free

reactions. Ethanolic KOH saponification was performed by adding to the cell-free reaction 1.0 ml of 40% potassium hydroxide (KOH) and 9.0 ml of 95% ethanol, adding a boiling chip, and boiling in a water bath at 90°C for 20 min.

For alkaline-pyrogallol saponification, the cell-free reaction to which cold TCA had been added was centrifuged for 5 min. at 27,000 x g in a Servall RC-2 refrigerated centrifuge and the supernatant discarded. The precipitate was suspended in 1.5 ml of 0.5% pyrogallol in methanol and 1.0 ml of 60% KOH. To this was added 1.5 ml of methanol and a boiling chip. The mixture was then refluxed for 50 min. When the TCA precipitation step was not desired, 1.5 ml of the 0.5% pyrogallol in methanol solution was added directly to the reaction mixture. The other two ingredients were then added as above and refluxing begun.

Dimethylsulfoxide (DMSO) extraction of the sterols was accomplished by suspending the TCA precipitate of the cell-free reaction in DMSO with the aid of an electric laboratory homogenizer. The precipitate suspended in 5.0 ml of DMSO was then heated at 100°C for 10 min. in an Arnold sterilizer. Alkali additions to the DMSO-protein suspensions were made following cooling of the heat treated solutions. The addition of 0.1 ml of 60% KOH was followed by vigorous shaking.

Sterols were extracted from the treated reaction mixtures with

three 10 ml portions of petroleum ether. If removal of the dissolved water from the extracts was desired, the three portions were combined and allowed to stand over approximately 2 g of anhydrous sodium sulfate for at least an hour. The extracts were then filtered by gravity through Whatman no. 1 filter paper. The petroleum ether was evaporated under a stream of nitrogen with gentle heating.

Extraction of sterols from whole cells

Three methods for extraction of the sterols from whole cells were used in the course of these experiments. KOH saponification was performed by adding to the sample of cell suspension (usually 2 to 3 ml) 1.0 ml of 40% KOH and a boiling chip. The mixture was covered loosely with foil and placed at 100°C for 5 hours in an Arnold sterilizer.

To treat the cells by acidic hydrolysis, an equal volume of 0.2N hydrochloric acid (HCl) was added to the suspension (making it 0.1N in HCl). This was then placed at 100°C for an hour in an Arnold sterilizer, after which the pH was adjusted to 10 using 60% KOH.

To perform alkaline-pyrogallol saponification, the cell suspension was centrifuged at 1500 r.p.m. for 5 min. in an International model EXD centrifuge. The supernatant was then discarded and the precipitated cells suspended in 1.5 ml of 0.5% pyrogallol in

methanol and 1.0 ml of 60% KOH. To this was added 1.5 ml of methanol and a boiling chip, and the mixture was refluxed for 50 min.

Sterols were extracted from the treated cell suspensions with three 10 ml portions of petroleum ether as described previously.

Analysis of sterols

When it was desired to determine the radioactivity of extracted sterols, the solvent extracts were placed directly into scintillation vials, the solvent evaporated under a stream of nitrogen, 10 ml of scintillation fluid added (0.3% PPO and 0.01% POPOP per liter of toluene) and then radioactivity assayed in a Packard Tri-Carb model 3000 liquid scintillation spectrometer (Packard Instrument Company, USA).

For colorimetric determination, the method of Fieser (1959) was used. The extracted sterols were dissolved in 3.0 ml of chloroform, and 2.0 ml of Liebermann-Burchard reagent (5% sulfuric acid in acetic anhydride) added with mixing. After allowing the color to develop at room temperature for exactly 20 min., the optical density was read at 625 mμ.

Digitonin precipitation

Digitonide derivatives of 3-β-hydroxy sterols were prepared by dissolving the sterols in 4.0 ml of acetone:absolute ethanol (1:1 v/v), then adding 4.0 ml of a solution of 0.5% digitonin in 50% ethanol and mixing. If carrier was required, 1.0 mg of ergosterol in solvent was added to the sterols, the solvent evaporated, and the procedure above begun. The samples were allowed to stand at 4°C overnight, then centrifuged at 1360 x g in an International model EXD centrifuge for 15 to 20 min. The precipitate was then washed twice with 5.0 ml portions of acetone:ether (1:1 v/v) and twice with 5.0 ml portions of ether. The washed precipitates were then dried at 80°C for 20 min. under nitrogen.

To break the digitonides and release the sterols, the procedure of Issidorides et al. (1962) was used. The dried digitonides were dissolved in 3 to 4 ml of DMSO by heating at 100°C for 10 min. This solution was then allowed to cool and extracted with one 10 ml aliquot and two 5 ml aliquots of hexane. The extracts were combined and allowed to stand over anhydrous sodium sulfate for 20 min., after which they were filtered through Whatman no. 1 filter paper and evaporated to dryness under nitrogen.

Chromatographic analysis of sterol mixtures

Thin layer chromatography was performed using 20 x 20 cm glass plates coated with a 0.25 mm layer of silica gel. The silica gel was impregnated with silver nitrate and the plates coated by adding 55 ml of a 4.5% silver nitrate solution to 25 g of silica gel in a mortar, mixing, and then applying to the plates with a Brinkmann-DeSaga spreader (Brinkmann Instruments Inc., USA). The solvent system used for chromatography was benzene:ethyl acetate (5:1 v/v). The separated components were detected by spraying the plates with Liebermann-Burchard reagent, then heating in an oven at 80°C for 20 min. If radioactivity of the separated components was to be determined, they were scraped from the plates into scintillation vials, 10 ml of scintillation fluid added, and radioactivity assayed in the Packard model 3000 automatic liquid scintillation spectrometer.

Purification of commercially obtained sterols

Commercially obtained sterols were recrystallized in absolute methanol before using as additives, carrier, or chromatographic standards.

RESULTS

Although there is a great volume of literature on the biosynthesis of ergosterol, there is no reference as to the possible methods for the regulation of ergosterol biosynthesis. This left an obvious area in which new studies on ergosterol biosynthesis could be begun.

It seemed logical that there should be some control over the synthesis of ergosterol by the cell, as a great amount of energy is required for sterol synthesis. It seemed unlikely that the cell would continue expending its energy synthesizing ergosterol under conditions in which there were excesses of that compound available to it.

As an approach to the problem of regulation, it was decided to look first for the two classical types of control which have been hypothesized: repression of enzyme synthesis and end-product inhibition of enzyme function. Either system could effectively cause the cessation of synthesis of ergosterol. The difference between the two can be seen in that end-product inhibition is an instantaneous effect, since it involves only the inhibition of an already present enzyme. A longer time is required for the effect of repression to be seen, since it involves an actual stoppage of enzyme synthesis.

With these theories in mind, a series of experiments was designed to determine whether or not end-product inhibition or repression is present in the sterol synthesizing system of yeast.

The experiments were designed to take advantage of the division of ergosterol biosynthesis into an anaerobic and an aerobic phase which can be achieved by the aforementioned anaerobic-aerobic shifts.

The first experiments were designed to test the effect of added yeast sterols on ergosterol biosynthesis in cells actively engaged in sterol biosynthesis. Yeast sterols were chosen as additives because the possibility existed that the inhibitory compound could be a sterol other than ergosterol. The predominant sterol in yeast is ergosterol, however. Yeast were grown anaerobically and then the sterol synthesizing system induced as described earlier by two hours of aeration. Following aeration, 25 ml of the resting cell suspension was placed in a 50 ml flask, the appropriate radioactive substrate added, and aeration continued at 30°C for another five hours. Yeast sterols had been added to the appropriate flasks beforehand by petroleum ether evaporation. Samples of 2 ml of this suspension were analyzed by KOH saponification for uptake of radioactive label into the non-saponifiable fraction. The results of this experiment can be seen in Table 1. The uptake of both the methyl group of methionine-methyl-C^{14} and of acetate-1-C^{14} was inhibited approximately 25% by the addition of yeast sterols.

Such results could indicate an allosteric inhibition, since the effect takes place in resting cells where there is no protein synthesis. The cell-free system was then examined, since a feedback inhibition

should also be expressed there if the sensitive enzyme is present and functioning. The cell-free system was prepared by grinding the frozen cells in a mortar and pestle, and the sterol additions to the reaction tubes made by petroleum ether evaporation. Analysis was by saponification in KOH and ethanol. The effect of yeast sterols on the uptake of the C^{14}-methyl group of methionine and of acetate-1-C^{14} into the nonsaponifiable fraction of the cell-free system is seen in Table 2. These results are somewhat different from those of the whole cell experiment, showing no effect on methyl-C^{14} uptake, and only a slight inhibition of acetate uptake.

One simple explanation can be offered for the differences in these results. Because the whole cells are being agitated, and because they quite possibly contain permeases for some of the sterols, some of the water insoluble sterol may get into the aqueous phase and be taken up by the cells. On the other hand, in the cell-free system there is no agitation, and because the sterols are so insoluble in water it is likely that most of the enzymes are never exposed to the added sterols.

These first results being encouraging that some type of feedback inhibition might exist, the experiments were repeated using a wider scope of yeast sterol concentrations. The whole cell experiments were performed in exactly the same manner as before with the single exception that analysis for label incorporation was done following 3

Table 1. Effect of added yeast sterols on sterol synthesis in resting yeast cells after five hours incubation with labeled substrate

Amount of added sterol	Radioactive substrate	Counts/min. incorporated	% of control
None (control)	Methionine-methyl-C^{14}	3, 943	--
40 μg	"	2, 991	76
80 μg	"	2, 935	75
None (control)	Acetate-1-C^{14}	293	--
40 μg	"	211	72
80 μg	"	223	76

Table 2. Effect of added yeast sterols on sterol synthesis in cell-free preparations of yeast

Amount of added sterol	Radioactive substrate	Counts/min. incorporated	% of control
None (control)	Methionine-methyl-C^{14}	16, 810	--
40 μg	"	17, 215	102
80 μg	"	17, 763	106
None (control)	Acetate-1-C^{14}	1, 294	--
40 μg	"	1, 080	83
80 μg	"	1, 273	98

hours of incubation with the radioactive substrates. As seen in Table 3, there was no inhibition of acetate uptake in this case, but methyl-C^{14} incorporation was again inhibited by greater than 20%. In the cell-free experiment performed exactly as before, again methyl-C^{14} was not inhibited by added sterols, and acetate uptake was inhibited slightly (Table 4).

The whole cell experiment as described previously was repeated twice more, this time using 50 ml of aeration medium per 20 g of wet cells. Following the 2 hours of aeration, 15 ml of the cell suspension was placed in 50 ml flasks, the different concentrations of added sterols having been added by petroleum ether evaporation. Incubation in the presence of labeled substrate was for 2 hours. Again the pattern is similar to that seen before (Table 5). Uptake of the methyl group of methionine was inhibited, but the uptake of acetate was not.

A possibility which had to be considered at this point was that there was an inhibition which was not being detected by analysis of the counts incorporated into the total sterol fraction. There could be an inhibition which did not change the total counts, but rather caused a buildup of a sterol other than ergosterol. In such a case the counts in the total sterol would remain the same, but their distribution in the different sterols would be altered. Separation of the different sterolic components of the total fraction and

Table 3. Effect of added yeast sterols on sterol synthesis in resting yeast cells after three hours incubation with labeled substrate

Amount of added sterol	Radioactive substrate	Counts/min. incorporated	% of control
None (control)	Methionine-methyl-C^{14}	6,530	--
10 μg	"	5,948	91
20 μg	"	4,636	71
40 μg	"	4,323	66
120 μg	"	5,074	78
None (control)	Acetate-1-C^{14}	482	--
10 μg	"	598	124
20 μg	"	506	105
40 μg	"	468	97
120 μg	"	664	138

Table 4. Effect of added yeast sterols on sterol synthesis in cell-free preparations of yeast

Amount of added sterol	Radioactive substrate	Counts/min. incorporated	% of control
None (control)	Methionine-methyl-C^{14}	29,567	--
20 μg	"	29,356	99
40 μg	"	31,377	106
120 μ g	"	27,943	95
None	Acetate-1-C^{14}	11,830	--
20 μg	"	10,035	85
40 μg	"	10,046	85
120 μg	"	11,404	96

analysis of the radioactivity of each should resolve this question. Sterols isolated from the previous whole cell experiment were separated by thin layer chromatography, the isolated components scraped from the plates and their radioactivity assayed. Only ergosterol and halfasterol (component 1 of Turner and Parks, 1965) were assayed. This was considered sufficient, since if the counts were shifted completely away from those sterols analyzed, such as a buildup of label in lanosterol, the effect would still be detected by the very low incorporation into the sterols analyzed as compared to the control. Table 6 shows the results. Similarly, a cell-free experiment was run with added sterols, the extracted sterols chromatogramed and the separated components assayed for radioactivity. Those results are in Table 7. Neither the whole cell nor the cell-free experiments show any significant shifting of the labeling pattern, thus discounting a possible "internal"effect.

Following these results, a series of experiments was done in which duplicate samples were taken throughout to test the consistency of the results. There were two whole cell experiments similar to those above run in this manner. In every case, the correlation between the duplicate samples taken was very poor.

Due to the erroneous results which were periodically received and the poor correlation seen betwen duplicate samples, it was decided that the method being used for extraction of sterols from whole

Table 5. Effect of added yeast sterols on sterol synthesis in resting yeast cells after two hours incubation with labeled substrate

Expr. No.	Amount of added sterol	Radioactive substrate	Counts/min. incorporated	% of control
1	None (control	Methionine-methyl-C^{14}	7,467	--
	25 μg	"	6,462	87
	50 μg	"	6,056	81
	None (control)	Acetate-1-C^{14}	9,117	--
	25 μg	"	10,393	114
	50 μg	"	12,027	132
2	None (control)	Methionine-methyl-C^{14}	989	--
	50 μg	"	635	64
	100 μg	"	851	86
	None (control)	Acetate-1-C^{14}	1,497	--
	50 μg	"	2,489	166
	100 μg	"	1,676	112

Table 6. Radioactivity of sterolic components of resting cells subjected to added yeast sterols as analyzed by thin layer chromatography

Spot	R_f	Identity by standards	Radioactive substrate	Reaction mixtures counts/min. in spot: Control no sterol	25 μg sterol	50 μg sterol
1	0.25	Ergosterol	Methionine	104	103	105
2	0.45	Halfasterol	"	103	99	111
1	0.25	Ergosterol	Acetate	106	103	99
2	0.45	Halfasterol	"	110	107	103

Table 7. Radioactivity of sterolic components of cell-free preparations subjected to added yeast sterols as analyzed by thin layer chromatography

Spot	R_f	Identity by standards	Radioactive substrate	Reaction mixtures counts/min. in spot		
				Control no sterol	50 μg sterol	100 μg sterol
1	0.25	Ergosterol	Methionine	125	127	107
2	0.45	Halfasterol	"	500	529	578
3	0.63	Lanosterol	"	82	74	86
4	1.00	Front	"	85	70	91
1	0.25	Ergosterol	Acetate	300	262	316
2	0.45	Halfasterol	"	96	76	72
3	0.63	Lanosterol	"	92	101	98
4	1.00	Front	"	63	96	94

cells was not adequate. The method of saponification being used was therefore examined for its efficiency in freeing sterols for extraction from the cell. In Table 8 are shown the results of an experiment in which our method of saponification was compared to two other methods for extracting sterols, alkaline-pyrogallol saponification and acidic hydrolysis, for the relative amounts of sterol extracted by each from whole cells. Cells were grown anaerobically in YMAF, aerated 2 hours in aeration medium, then methionine-methyl-C^{14} added and aeration continued for 5 hours more. The cells were then washed, suspended in 100 ml of cold distilled water, and duplicate 5 ml samples analyzed for Liebermann-Burchard positive sterols and methyl-C^{14} uptake for each method. Alkaline-pyrogallol proved to be by far the most efficient method for extracting sterols from whole cells.

Table 8. Comparison of three methods for extracting sterols from whole cells of yeast

Treatment	Average counts/min per 5 ml cell suspension	Average μg sterol per 5 ml cell suspension
KOH saponification	125,489	350
Acidic hydrolysis	55,716	124
Alkaline-pyrogallol	238,464	653

With this improved method for analyzing sterol content in whole cells, it was hoped that a more definitive answer could be found concerning the regulation of sterol synthesis in yeast. An attempt was made to achieve this by analyzing the uptake with time of labeled substrates into sterol in resting cells subjected to various levels of added sterols. The first experiment of this type was designed to demonstrate feedback inhibition. Anaerobically grown cells were aerated 2 hours in aeration medium, then 75 ml of suspension transferred to 250 ml flasks to which had previously been added different concentrations of yeast sterols by petroleum ether evaporation. The control flask had no added sterols. To each flask was added 1.0 μc of acetate-1-C^{14}, and aeration at 30°C continued for 19 hours more. Samples of 3 ml were taken at intervals for sterol analysis. Figure 1 shows the uptake of the radioactive label into the nonsaponifiable fraction as a function of time for the different reaction flasks. According to these results, there does not appear to be any feedback inhibition of sterol synthesis. There

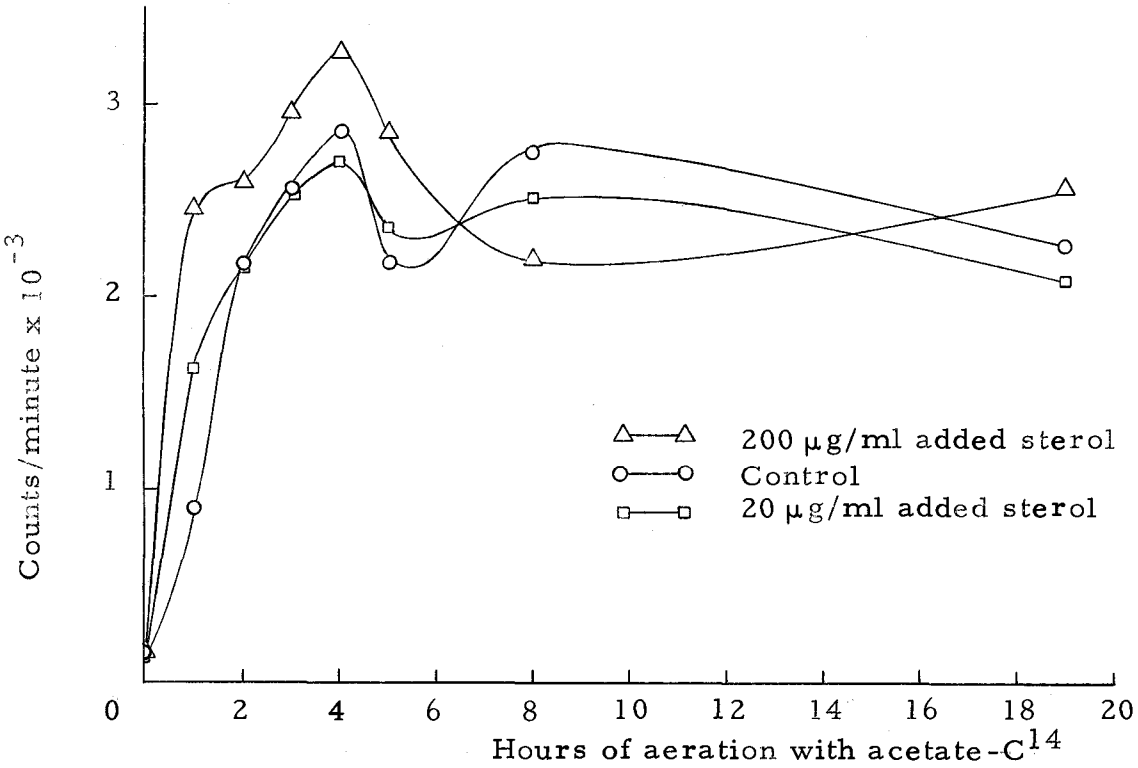


Figure 1. The effect of exogenous sterol on the time-course incorporation of acetate-1-C^{14} into sterol by resting cells after two hours of aeration

appears to be a stimulation of uptake by the addition of 200 μg sterol per ml; however, it should be noted that the fluctuations in uptake in the different flasks become out of phase, causing a change in the apparent results with time. That the fluctuations in uptake of the label are real, and not due to experimental error, has been shown by Parks and Starr (1963), and is further substantiated in this work.

Another possible point of control which was considered was the activation step. As has been stated previously, aeration of anaerobically grown yeast cells activates certain enzymes involved in the terminal steps of sterol biosynthesis. It was decided to test the effect of added sterols on this activation step for a possible controlling role. The procedure was the same as in the previous experiment, with the exception that aeration in the presence of added sterol and labeled acetate was begun immediately following harvesting of the anaerobically grown cells. Thus, activation must occur in the presence of exogenous yeast sterols. The results (Figure 2) again show no obvious effect caused by the added sterols. Again, however, the fluctuations of uptake and differences in phase of fluctuations may be seen.

During the course of these experiments, it was discovered in these laboratories that a yeast extract solution would bind sterols added to it in alcohol solution. The sterols were bound such that they could not be extracted from the aqueous mixture with organic

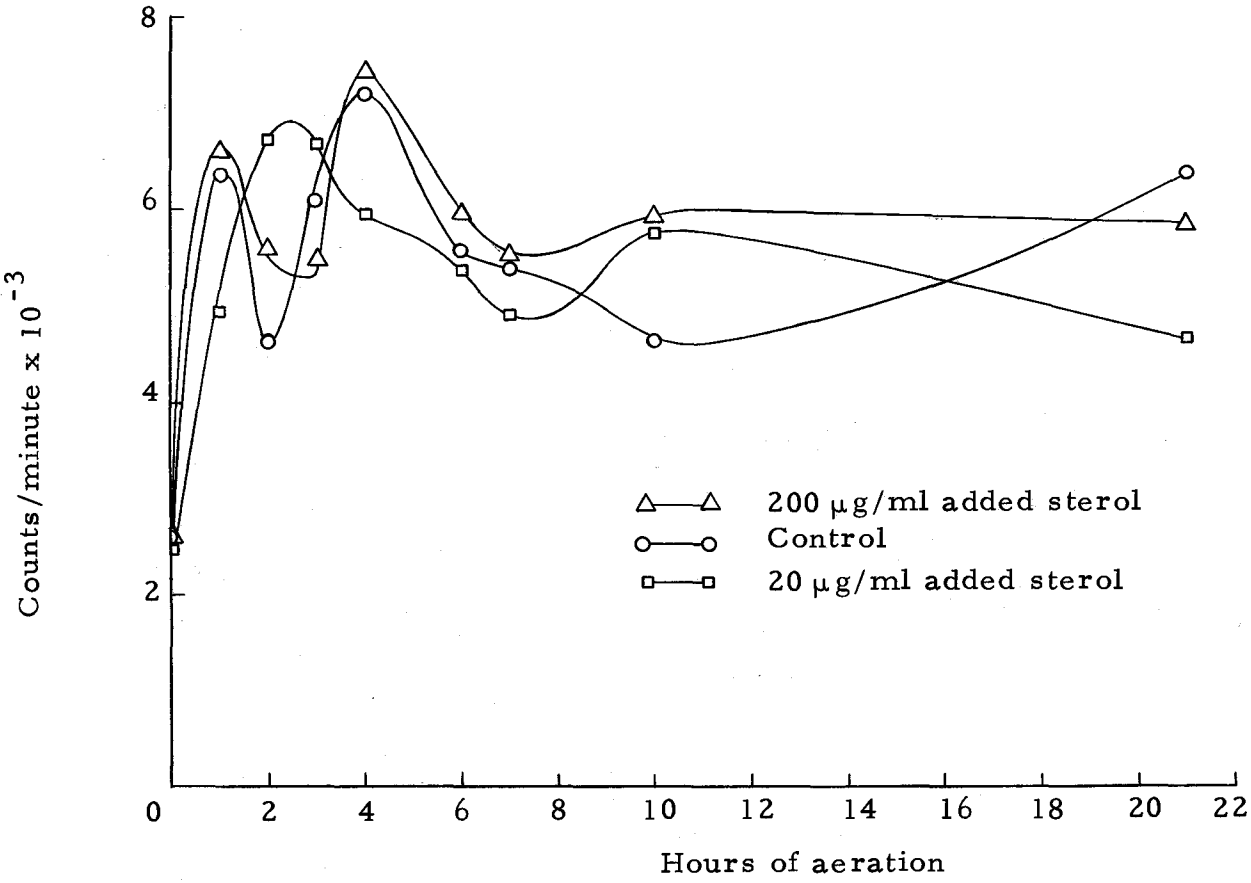


Figure 2. The effect of added yeast sterols on the incorporation of acetate-1-C^{14} into sterol by resting cells during the initial hours of aeration

solvents (Adams and Parks, 1967a). Because the complexing agent occurred naturally in yeast, it was assumed that the sterol complex would be physiologically active. This was later proved true (Adams and Parks, 1968). The discovery provided a means for adding sterol to the reaction mixtures in these studies on control of ergosterol biosynthesis. The water soluble complex assured both an even distribution of the sterol in the reaction system, and a soluble form of the sterol which might be more available to the cell.

The test for the activation of the sterol synthesizing enzymes was therefore repeated using the yeast extract-sterol complex as a means for adding the sterol. Approximately 40 g of wet cells from anaerobic cultures was washed, divided into three equal portions, and each of the fractions placed in 65 ml of aeration medium consisting of 1% glucose and 5% yeast extract. Prior to adding the cells, ergosterol in a 95% ethanol solution was added to the test medium in the desired concentration. To the control was added an equal volume of 95% ethanol. Placing the flasks at 100°C in an Arnold sterilizer as described previously removed the ethanol. Following cooling, the cells and 1.0 μc of acetate-1-C^{14} were added to each flask and aeration begun. Samples of 3 ml were taken at intervals for analysis of uptake of label into the sterol of the cells. Figure 3 shows the uptake of label by a control flask, one with 15 μg ergosterol per ml, and one with 150 μg ergosterol per ml. In this case there does appear to be

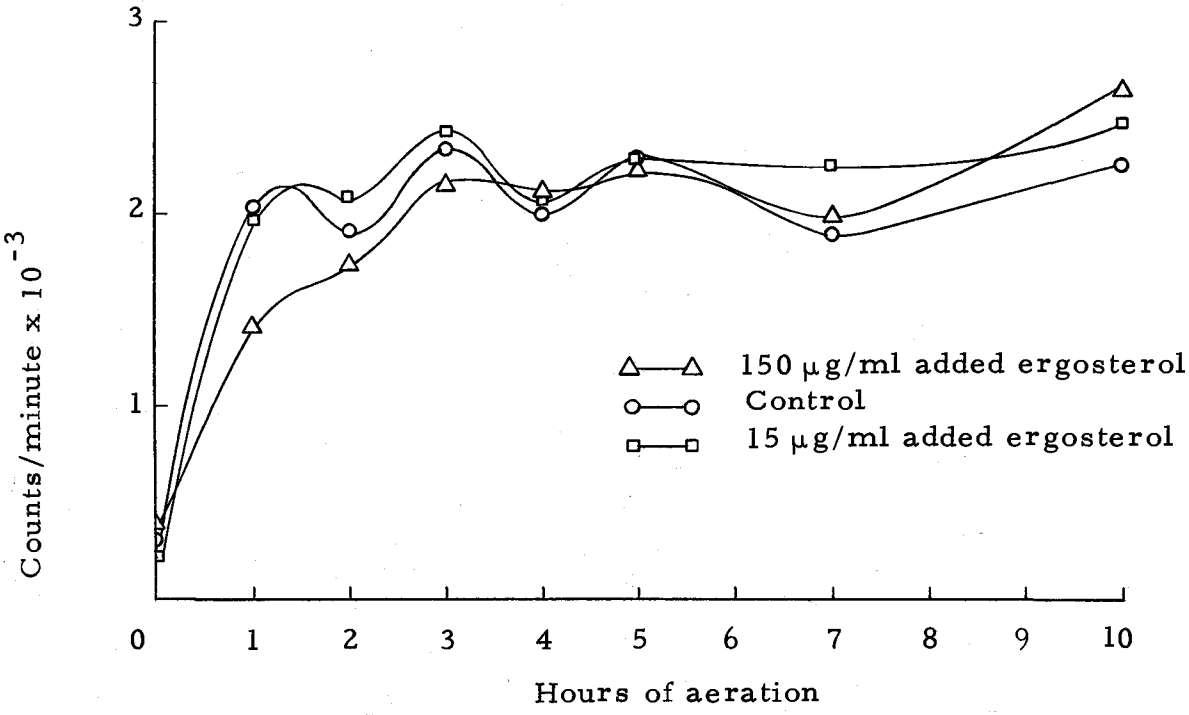


Figure 3. The effect of the addition of sterol as a yeast extract complex on the whole cell incorporation of acetate-1-C^{14} into sterol

an inhibition of acetate uptake initially caused by 150 μg per ml added ergosterol.

A similar experiment was performed in which the incorporation of the methyl group of methionine into the sterol of the cells was observed. The aeration medium in this case contained 1% glucose and 0.5% yeast extract. The lower concentration of yeast extract was used because earlier experiments had shown that higher concentrations of yeast extract inhibited uptake of the methionine label. Ergosterol additions were made as before. One flask was a control, and two flasks contained 150 μg ergosterol per ml. The phenomenon is observed in this experiment, as in the previous ones, whereby the fluctuations in uptake of the label in the flasks containing added ergosterol appear to be shifted somewhat out of phase with those of the control (Figure 4). Correlation between duplicate flasks was again poor.

A new series of experiments was initiated on the regulation of sterol synthesis with the discovery that the sterol complexing agent in yeast extract was a polysaccharide which could be separated from a yeast extract solution by passing it through a column of Sephadex G-50 (Adams and Parks, 1968). The isolated complex solution could be lyophilized, and the endogenous sterol in it determined. Using the lyophilized complex, controlled experiments could be done in which there would be no side effects due to the other

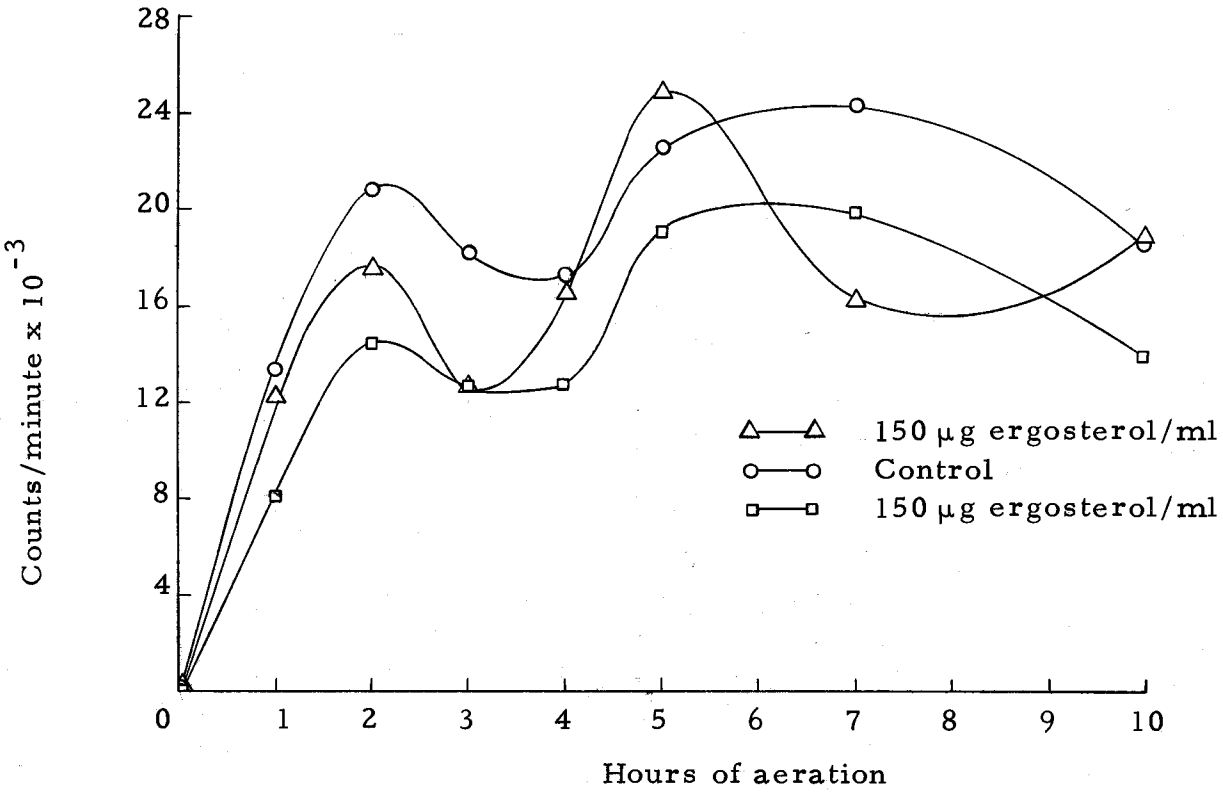


Figure 4. The effect of the addition of sterol as a yeast extract complex on the incorporation of methyl-C^{14} into sterol by resting cells

components in the yeast extract, and in which the same amounts of polysaccharide and sterol could be added each time. Three experiments were designed using this more closely controlled system which together, it was hoped, would demonstrate positively the presence or absence of any end-product control.

The first experiment tested for feedback inhibition. Cells grown anaerobically in YMAF medium were harvested, washed, and approximately 10 g of wet cells placed in 50 ml of each of three different aeration media. One of these was a control consisting of the normally used aeration medium. One was a control for the complex, containing in addition to the glucose and the phosphate 0.108 g of the lyophilized complex, which is almost completely free of sterol. The third medium contained 0.108 g of a lyophilized complex obtained from Sephadex treatment of a 15% yeast extract solution to which had been added ergosterol. It contained 2.32 μg sterol per milligram, giving the medium a concentration of 5 μg ergosterol per ml. Aeration was continued for 2 hours at 30° C; then cell-free extracts were prepared from each of the three cell suspensions using the Eaton press. The reaction mixtures contained the usual ingredients. Four reactions were run using each preparation, one reaction of each being stopped at intervals over a 5 hour period by addition of 0.3 ml of 10% TCA.

Radioactivity incorporated into sterol from methionine-methyl-C^{14} was determined by DMSO analysis. Protein determinations were according to the method of Lowry, et al. (1951). The results are in Figure 5. It appears that either the polysaccharide itself inhibits enzyme activity to a small extent (15%), or that the small amount of sterol present in the "sterol free complex" is enough to cause the observed inhibition, and that any additional sterol has no further effect.

In the second experiment of this series the procedure followed for preparation of the different enzymes was the same as for the previous experiment. However, in addition to the usual substrates in the cell-free reaction, to the control reactions was added 0.1 ml of distilled water. To the enzyme reaction from the cells aerated in sterol-free complex was added 0.1 ml of a solution of 0.108 g of sterol free complex per 5 ml. And to the reactions prepared from cells aerated in the presence of ergosterol complex was added 0.1 ml of a 0.108 g per 5 ml solution of that complex. Time course analysis was done as in the previous experiment. In Figure 6, it can be seen that the results are quite different from those received previously. Addition of ergosterol this time seemed to stimulate uptake of the methyl-C^{14} label.

For the last experiment of this series, two cell-free extracts were prepared. To one flask of YMAF was added as an ethanol

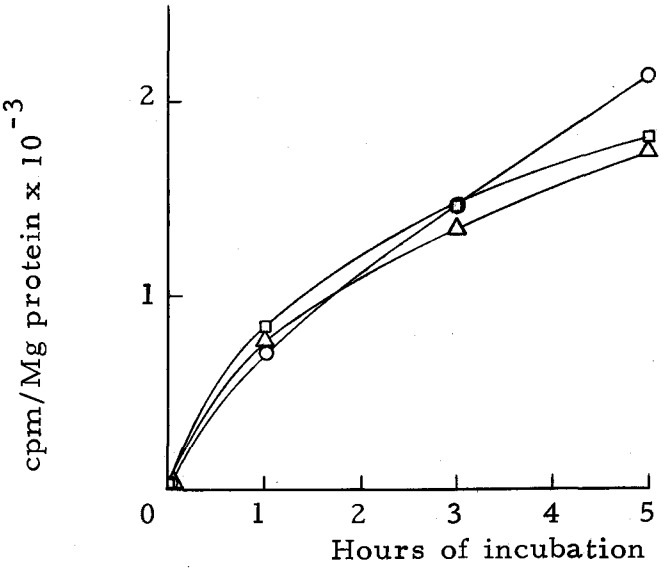


Figure 5. The cell-free synthesis of sterols by extracts from cells activated in the presence or absence of ergosterol complex. Control o—o. Complex control □—□. Ergosterol complex △—△.

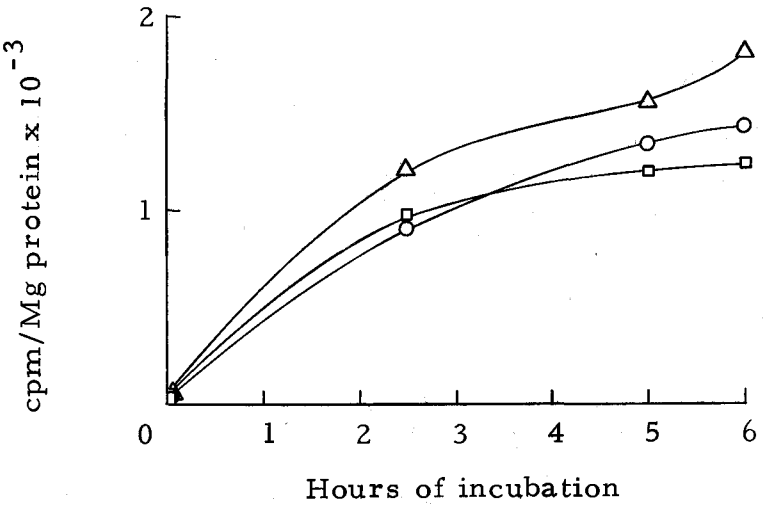


Figure 6. The effect on cell-free sterol synthesis of activation of cells and incubation of the resulting extracts in the presence or absence of ergosterol complex. Control o—o. Complex control □—□. Ergosterol complex △—△.

solution prior to autoclaving 5 μg of ergosterol per ml of medium. This was grown statically as usual, then the cells harvested and aerated in sterol containing complex as in the previous two experiments. After aeration, extracts were prepared from the cells. This extract was used in a series of reactions, half of which were controls with water added, and the other half having ergosterol complex solution added as before. At the same time, a second flask of YMAF was inoculated with yeast and grown and aerated normally. The extract prepared from these yeast was used in a series of reactions treated exactly as above. A time course analysis of the uptake of the methyl-C^{14} label was performed on these reactions, the results of which are shown in Figure 7. Again there is no obvious inhibitory effect.

From these studies it appeared that there was no feedback inhibition of sterol biosynthesis caused by ergosterol, and that ergosterol did not have any effect on activation of the enzymes involved in sterol synthesis. Furthermore, anaerobic growth in the presence of ergosterol did not appear to affect the sterol synthesizing system in any way. It was therefore thought that any effect exogenous sterol might have on sterol biosynthesis might be more likely to be exhibited in aerated growing cells, in which exogenously supplied ergosterol should supplant the need for the cell to synthesize its own. Two 2-liter flasks were used, each containing

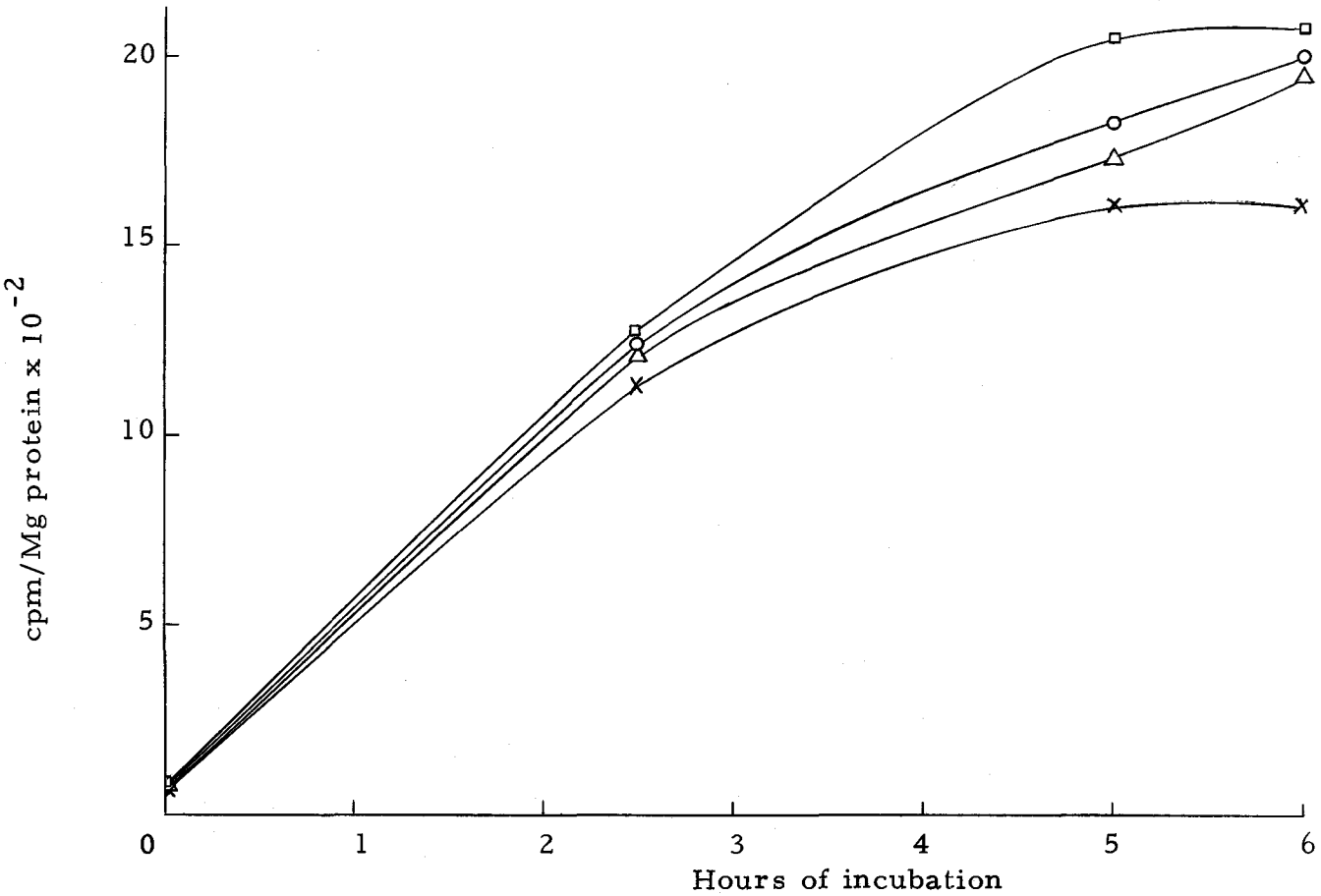


Figure 7. A test for feedback inhibition by ergosterol of cell-free extracts from cells grown anaerobically and activated in the presence or absence of ergosterol. Extracts from control cells incubated normally (○—○), and with ergosterol (□—□). Extracts from ergosterol grown cells incubated normally (△—△), and with ergosterol (×—×).

500 ml of TCA medium. To one was added ethanolic ergosterol solution to a concentration of 150 μg per ml, to the other the same amount of 95% ethanol. Autoclaving sterilized the medium and at the same time removed the ethanol. These flasks were inoculated with 0.5 ml of cell suspension from a seed flask, and incubated at 30°C for 40 hours on a rotary shaker. Then, 200 ml of the suspen- from each flask was harvested, the cells washed twice, then sus- pended in 75 ml of aeration medium. Following the addition of 1.0 μc of methionine-methyl-C^{14}, the suspensions were aerated for 8 hours at 30°C. At intervals 3 ml samples were taken for analysis by alkaline-pyrogallol saponification. Although again the phase shift is seen, there is no indication of retardation of sterol synthesis due to the added ergosterol (Figure 8).

To further test for any possible effect caused by the added erg- osterol, the remaining 300 ml of cell suspension in each case was harvested, washed twice, then cell-free extracts prepared from the cells by breaking in the Eaton press. The incorporation of methyl- C^{14} into the nonsaponifiable fraction, as determined by alkaline- pyrogallol analysis, was determined for each enzyme, and activity per milligram of protein calculated. Table 9 shows that there was 28% less activity in the enzyme from cells grown in the presence of added ergosterol.

A repeat of the cell-free part of this experiment was performed

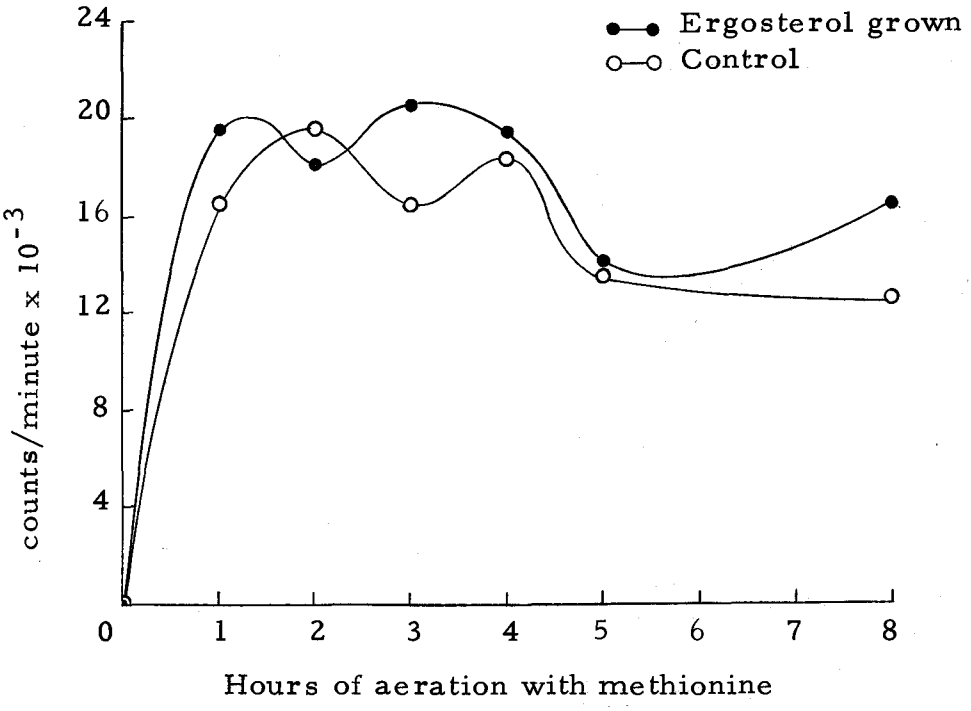


Figure 8. The incorporation of methyl-C^{14} into sterol by resting suspensions of cells grown aerobically for 40 hours in the presence or absence of added ergosterol

Table 9. Activities of cell-free sterol synthesizing preparations of yeast from cells grown 40 hours aerobically in the presense and absence of ergosterol

Cells from which enzyme prepared	Incorporation: counts/min./mg protein	% repression of activity
Control cells	48.2	--
Ergosterol-grown cells	34.6	28

in which four 2 liter flasks containing 500 ml TCA each were used. Two were test flasks containing 150 μg ergosterol per ml of medium, added as before, and two were control flasks. After 30 hours of aerobic growth at 30°C, the cells from like flasks were combined, harvested, washed twice, and cell-free extracts prepared using the Eaton press. The incorporation of methyl-C^{14} into sterol, as determined by alkaline-pyrogallol saponification, is shown in Table 10. The activity of the enzyme from the ergosterol grown cells is again less than the controls, in this case by 14%.

The experiments up to this point had given only very inconclusive results. The existence of some type of end-product governed control could be neither verified nor denied. The cell-free experiments using aerobically growing cells seemed to give the most positive results. But, even there, when the experiment was tried for a third time there was no difference in the activities of the two enzymes. The three basic methods used thus far were to analyze

either the total sterol accumulated in resting whole cells, the time course accumulation of label in resting cells, or the activity of cell-free preparations subjected to different conditions.

A new approach which seemed that it might offer some insight into the problem was to determine the effect of ergosterol on the rate of sterol synthesis at different times during the aerobic growth of yeast cells. The results of such an experiment are seen in Figure 9. Three 2 liter flasks each containing 500 ml of TCA medium were used. One served as a control, one contained 20 μg per ml of added ergosterol, and the third had 200 μg per ml added ergosterol. Ergosterol additions were made as an ethanol solution, as usual. These were inoculated with approximately 10^6 cells per ml from a seed flask and placed at $30^{\circ}C$ on a rotary shaker. Rate determinations were made by removing, at the appropriate times, two 10 ml samples from each of the growth flasks into 50 ml flasks, also on the shaker. Methionine-methyl-C^{14}, 1.0 μc, was added to each 50 ml flask and incubation continued for 4 hours. Cell numbers were determined at the beginning and end of each 4 hour incubation period using the Coulter counter. The sterols were extracted using alkaline-pyrogallol saponification. Rates were calculated as the counts per minute incorporated per 10^6 cells per 4 hours. As can be seen, during the early log phase the rates of sterol synthesis are lower in the flasks with added sterol.

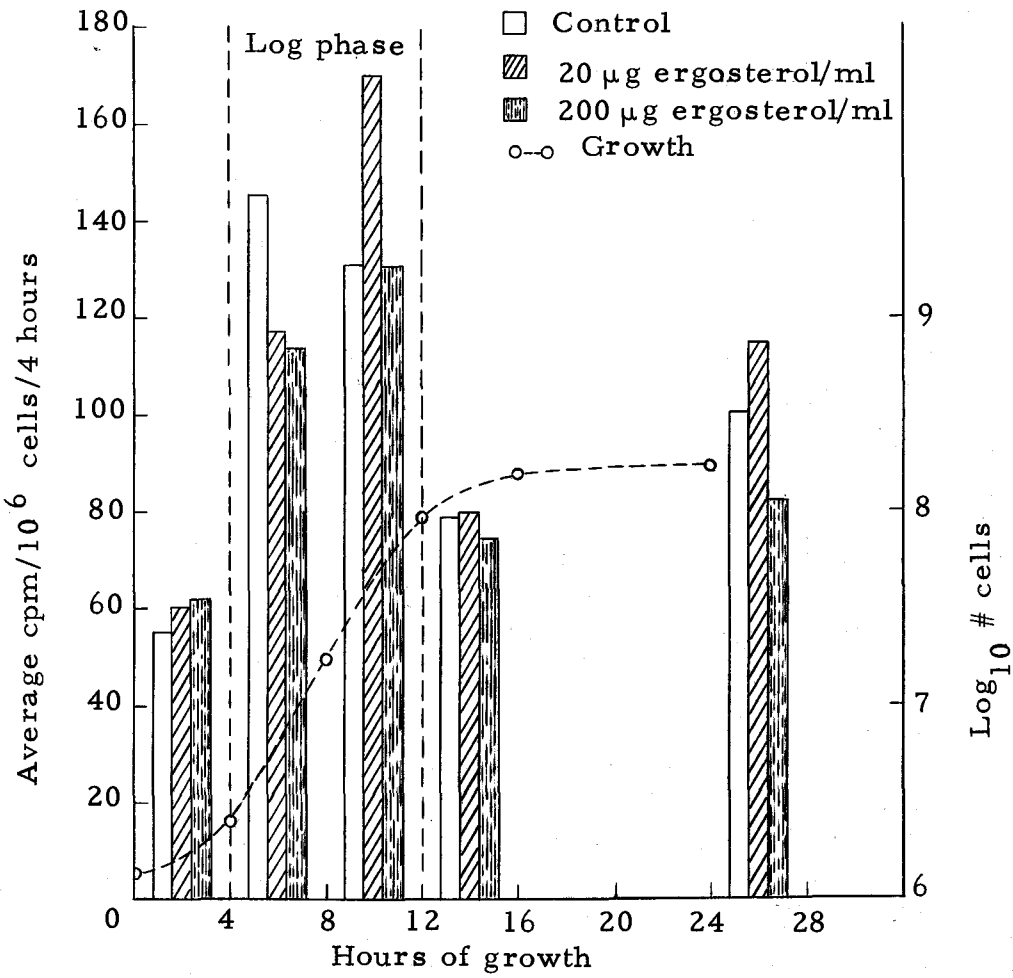


Figure 9. The influence of added ergosterol on the rate of sterol synthesis during different stages of aerobic growth of yeast

Both the previous cell-free experiments using enzyme from cells grown aerobically in the presence of added sterols and this experiment showing the relative rates of sterol synthesis during growth in the presence of added sterols imply that there are fluctuations in the relative rates of synthesis. It was therefore decided to test again for such a time factor involvement. Three flasks containing 500 ml of TCA each, a control, one with 20 μg ergosterol per ml, and one with 200 μg ergosterol per ml were inoculated with 10^6 cells per ml and grown as before. The cells were grown 26 hours. At this stage, according to Figure 9, there should be a lower sterol synthesizing activity in the flask with 200 μg ergosterol per ml than in the control. The flask with 20 μg ergosterol per ml should have a higher activity than the control. Cell-free preparations were made from the cells from each flask, and duplicate reactions run with each using incorporation of the methyl-C^{14} label of methionine as a measure of sterol synthesis. Table 11 shows that the activities were exactly as would be predicted from the whole cell experiments.

As stated earlier, with the discovery that yeast extract would complex sterol, this method was turned to as a means for adding sterol to aqueous solutions for the sterol inhibition studies. That yeast extract in high concentrations inhibited the uptake of methionine label into sterol was first noticed in studies of incorporation

Table 10. Activities of cell-free sterol synthesizing preparations of yeast from cells grown 30 hours aerobically in the presence and absence of ergosterol

Cells from which enzyme prepared	Incorporation: counts/min. /mg protein	% repression of activity
Control cells	46.3	--
Ergosterol-grown cells	39.4	14

Table 11. Activities of cell-free sterol synthesizing preparations from yeast cells grown 26 hours aerobically in the presence and absence of ergosterol

Amount of sterol in which cells grown	Counts/min. /mg protein	% of control
None (control)	460	--
20 μg per ml	496	108
200 μg per ml	264	57

of the label by whole cells. It was noticed that in a suspension of the cells in a 5% yeast extract solution the amount of label incorporated seemed to be reduced. Then, it was observed in a cell-free preparation that adding 0.5 ml of a 2% yeast extract solution inhibited the incorporation of the methyl-C^{14} group by 60%. It was decided that the effect merited further examination.

Fractionation of yeast extract solution on a column of Sephadex G-50 showed that the inhibition resided in a small molecular weight component. Almost all of the inhibitory activity is in the later

fractions coming off the column. Table 12 illustrates this. Two ml of a 5% yeast extract solution was passed through a column of 10 ml of Sephadex G-50 equilibrated with distilled water. Four fractions of 2-3 ml were collected and tested for their effect on the cell-free incorporation of methyl-C^{14} into sterol. As another control, a portion of 5% yeast extract was dialyzed against distilled water until no more yellow color was imparted in the water. The detection of complex formation was performed by adding to 1.0 ml of each of the fractions 0.1 ml of a solution of 0.5 mg ergosterol per ml of 95% ethanol. Formation of crystals of sterol floating on the surface of the sample indicated no complex formation.

Table 12. Inhibition of methionine-methyl-C^{14} incorporation in cell-free preparations by yeast extract fractions from a Sephadex G-50 column

Addition to cell-free reaction	Complex formation	Counts/min./mg protein	% incorporated compared to control
0.2 ml H_2O	−	109	--
0.2 ml 5% yeast extract	+	72	66
0.2 ml fraction #1	+	111	108
0.2 ml fraction #2	±	55	51
0.2 ml fraction #3	−	63	58
0.2 ml fraction #4	−	162	149
0.2 ml dialyzed yeast extract	+	172	158

Further and more exacting separations proved that the inhibitory activity resided in a low molecular weight fraction. Further tests also showed that the inhibitor fraction could not complex sterols. To determine the scope of the inhibition, the effect of the inhibitor on the incorporation of acetate-1-C^{14} into sterol by cell-free preparations was tested. It was found that the addition of 0.2 ml of an inhibitor fraction from Sephadex treated yeast extract caused a 40% reduction of incorporation of the label. Since both acetate and methionine incorporation are affected, it may be asked whether or not several enzymatic steps are inhibited.

The inhibitor fraction was ashed to determine whether or not the inhibitor is organic in nature. Five ml of the inhibitor solution isolated from a Sephadex column was placed in a 50 ml beaker and evaporated to dryness on a hot plate. The residue was placed in a muffle furnace at 600°C overnight. After cooling, the ash was dissolved in 5 ml of 0.1 N HCl. The pH of the solution was approximately 5 following this procedure, indicating a high buffer capacity. A phosphate determination was performed on this solution using the Fiske-Subbarow method (Fiske and Subbarow, 1925). A value of 500 μg phosphorus per ml was received.

A standard cell-free preparation was made, and a second preparation made using 0.1M Tris buffer at pH 6.5 in place of the phosphate buffer normally used. Using these two cell-free

preparations, the ashed inhibitor solution and a solution of 500 μg phosphate phosphorus per ml were tested for their effect on the ability of the extracts to incorporate methyl-C^{14} into sterol. Also, the ability of the extracts to incorporate S-adenosylmethionine-methyl-C^{14} (AM) label under these conditions was tested. This was to determine whether or not the inhibitor affects the AM synthetase reaction. The results are seen in Table 13.

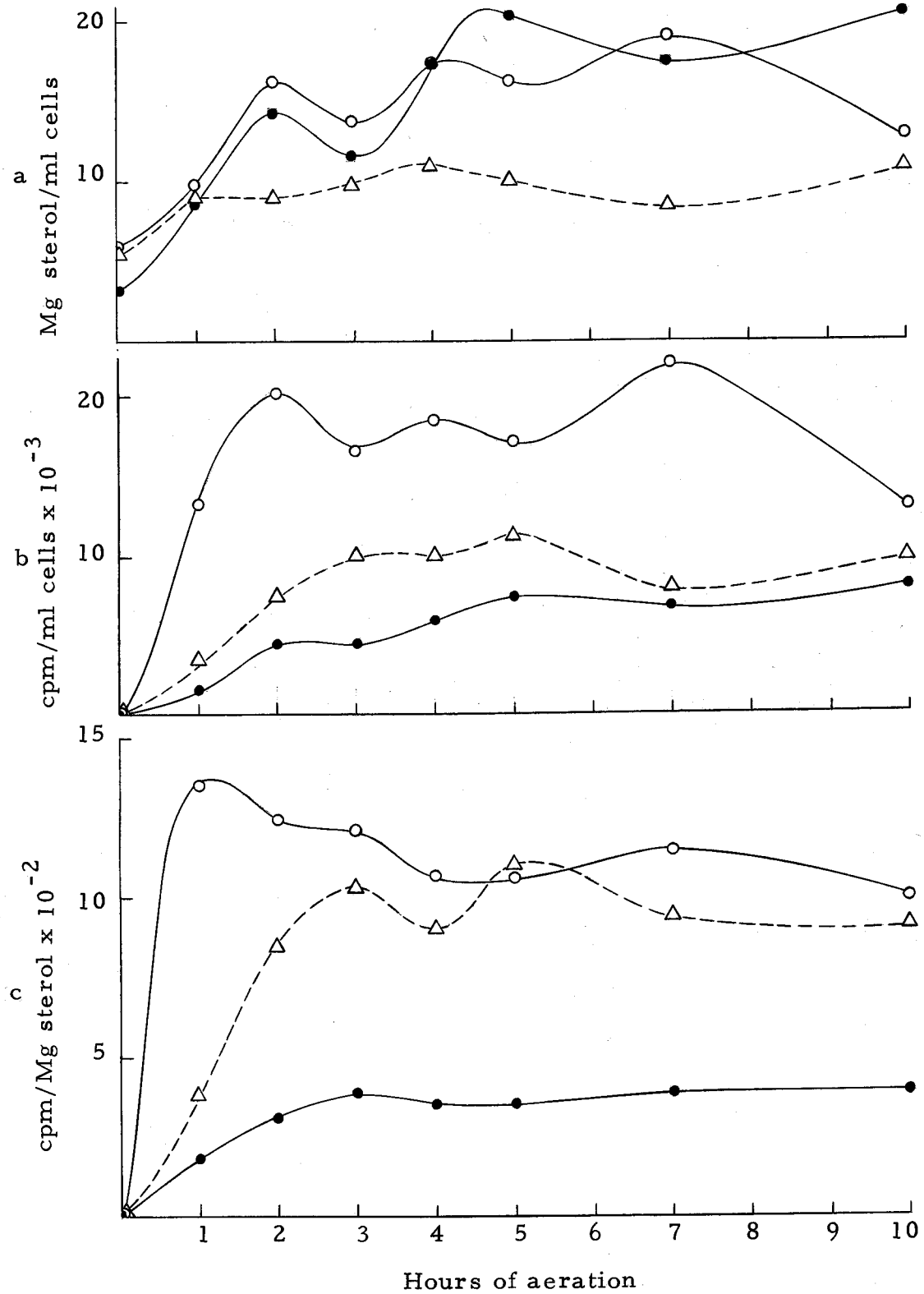
Table 13. Comparison of ashed inhibitor and standard phosphate for their effect on methionine and S-adenosylmethionine incorporation in cell-free systems

Addition to cell-free reaction	Radioactive substrate	Buffer	Counts/min./mg protein	% inhibition
0.2 ml H_2O	Methionine (1.0 μc)	PO_4	369	--
0.2 ml ashed inhibitor	Methionine (1.0 μc)	PO_4	108	71
0.2 ml standard PO_4	Methionine (1.0 μc)	PO_4	331	10
0.2 ml H_2O	AM (0.0021 μc)	PO_4	33	--
0.2 ml ashed inhibitor	AM (0.0021 μc)	PO_4	27	18
0.2 ml standard PO_4	AM (0.0021 μc)	PO_4	33	none
0.2 ml H_2O	Methionine (1.0 μc)	Tris	277	--
0.2 ml ashed inhibitor	Methionine (1.0 μc)	Tris	64	77
0.2 standard PO_4	Methionine (1.0 μc)	Tris	252	9
0.2 H_2O	AM (0.0021 μc)	Tris	31	--
0.2 ashed inhibitor	AM (0.0021 μc)	Tris	23	26
0.2 standard PO_4	AM (0.0021 μc)	Tris	31	None

From these results it is obvious that the ashed inhibitor solution does retain the inhibitory activity, and that phosphate is not the component causing the inhibition. The incorporation of AM is inhibited by about 20%, which is significant, but it is not as drastic an effect as seen with methionine. The results using AM may not be accurate because of the low level of radioactivity added.

An ion which exhibits a large inhibitory effect on sterol synthesis in whole cells (Parks and Starr, 1963) and also inhibits transmethylation in these cell-free preparations (Parks, Turner and Larson, 1965), is cupric ion. Although there should be no large concentrations of this ion in yeast extract, it was decided to compare the inhibitory activities of cupric ion and the yeast extract inhibitor.

Approximately 20 g wet cells, grown anaerobically in YMAF, were harvested, washed twice, and suspended in 200 ml of aeration medium. In each of three 250 ml flasks was placed 65 ml of the cell suspension. To one of each of the three was added 3.0 ml H_2O, 3.0 ml yeast extract inhibitor solution, and 3.0 ml copper sulfate solution to a concentration of 0.13 mg copper sulfate per ml of suspension. Aeration was begun and samples of 3.0 ml taken from each flask at intervals for analysis by alkaline-pyrogallol saponification. A portion of the sterols extracted in each case was analyzed for radioactivity, and the remainder analyzed for Liebermann-Burchard positive sterols. The results are shown in Figure 10.


a
Mg sterol/ml cells
20
10
b
cpm/ml cells x 10^{-3}
20
10
c
cpm/Mg sterol x 10^{-2}
15
10
5
1
2
3
4
5
6
7
8
9
10
Hours of aeration

It can be seen that the inhibitor lowers the uptake of methyl-C^{14} more than copper sulfate. However, the copper sulfate also lowers the levels of Liebermann-Burchard positive sterols considerably, while the inhibitor does not. The summation of these effects can be seen in Figure 10 (c), where the radioactivity per microgram of sterol is plotted versus time. There it can be seen that the inhibitor has actually lowered the specific activity of the sterols in the cells. This value has not varied from that of the control in the case of the copper sulfate treated cells. This can be interpreted to mean that the copper sulfate causes a general inhibition which lowers total sterol synthesis, whereas the inhibitor only affects transmethylation, causing a buildup of Liebermann-Burchard positive material. The results argue that the inhibitor from yeast extract is not cupric ion.

A final test was to ascertain that the inhibitor is indeed cationic in nature. Five ml of the inhibitor isolated by Sephadex treatment of yeast extract was passed by batch process through 20 ml of Dowex-50, a cation exchanger in the hydrogen ion form. The fraction recovered was tested for inhibitory activity in a cell-free system (Table 14).

From the results it appears that most of the inhibitory activity does reside in the cationic fraction of the solution.

During the course of these studies, a recurring problem has been the inconsistency of the results obtained, even among duplicate

Table 14. Test for inhibition of cell-free incorporation of methionine by inhibitor treated with a cation exchange resin

Reaction #	Addition	Counts/min.	% Inhibition
1	0.2 ml H_2O	109,992	--
2	0.2 ml inhibitor solution	17,844	84
3	0.2 ml Dowex treated inhibitor	96,094	13
4	0.2 ml H_2O	109,777	--
5	0.2 ml inhibitor solution	17,377	84
6	0.2 ml Dowex treated inhibitor	85,110	22

samples, using the conventional methods of saponification and extraction for sterol isolation and analysis. This has led to the conclusion that with these methods one is only extracting a portion of the total sterol, and that that fraction is variable. The aforementioned discovery of a water soluble form of sterol in yeast and the elucidation of some of the properties of the solubilizing agent have offered some possible answers to the problem and suggested a new method for extracting sterols from the cell-free system used in this study (Adams and Parks, 1967a, 1968).

One of the pertinent properties of the solubilizing agent was that it is not destroyed by saponification. Because the sterol cannot be readily extracted from the complex by organic solvents (Adams and Parks, 1967a), the possibility was suggested that even after saponification some of the sterol may remain bound in the complex,

and therefore be unextractable. The extreme ease of formation of the aqueous sterol complex suggested a second possibility. If the sterol is bound to the protein in the cell-free reaction, then its release by saponification into the solution may only result in its being complexed to the solubilizing agent endogenous to the system. In either case, the end result is nonextractability of part of the sterol through binding in the soluble complex. From these findings it was apparent that a new method of extraction of the sterolic components of these systems was needed.

For the development of this procedure, in all cases the incorporation of methionine-methyl-C^{14} by the cell-free preparations has been used as a measure of sterol synthesis.

It was known that TCA did not precipitate the soluble complex. Therefore, since the results in Table 15 show that virtually all of the methyl-C^{14} label from methionine-methyl-C^{14} incorporated into the nonsaponifiable fraction is found in the TCA precipitable fraction of the cell-free reaction, it appears that the ergosterol precursors are bound to protein, rather than being free. However, the possibility exists that the precursors still remain in the soluble form as a protein-sterol-polysaccharide complex.

It was decided that an ideal method for extraction of the sterols would be one which would both release the sterol from the precipitated protein and free it from the complex. If these conditions were

Table 15. Comparison of saponification of supernatant and precipitate from TCA precipitation of cell-free extracts

Method of extraction	Experiment #	Counts/min. extracted
Saponification of supernatant	1	121
	2	117
Saponification of TCA precipitate	1	13, 311
	2	14, 728

met, then the free sterol should be extractable with some nonpolar solvent, such as petroleum ether. Since the binding of sterol in the complex appeared somewhat analogous to the association of sterol and digitonin in digitonides, DMSO was decided upon as a likely reagent. It was known to break digitonides, leaving the sterol free and extractable (Issidorides, et al., 1962), and it was reasoned that its extreme penetrating ability might also make it ideal for releasing the sterol from the protein.

In Table 16 are shown the results obtained when three methods of extraction were attempted: saponification of the cell-free reaction mixture; saponification of the TCA precipitate of the cell-free reaction mixture; and suspension of the TCA precipitate of the reaction mixture in DMSO. It can be seen that saponification of the TCA precipitate released approximately twice the counts that saponification of the entire reaction mixture did, and that DMSO treatment of the TCA precipitate released over three times as many extractable

Table 16. Dimethylsulfoxide treatment of the TCA precipitate compared to saponification of entire cell-free reaction and TCA precipitate of cell-free reaction

Tube #	Method of extraction	Counts/ min. extracted.
1	Saponification of reaction mixture	4,590
2		6,169
3	Saponification of TCA precipitate	12,716
4		8,531
5	DMSO of TCA precipitate	35,783
6		32,663

counts as saponification of the TCA precipitate.

At the low pH caused by the TCA precipitation, it seemed likely that this DMSO method would extract a variety of methylated lipids in addition to the sterols. The results of adding base to the protein-DMSO suspension to a pH of approximately 9, after heating, as compared with straight DMSO treatment of the TCA precipitate and saponification of the reaction mixture are shown in Table 17. The addition of alkali reduced by 40% the amount of labeled material extracted by DMSO treatment, but this amount was still over two and one-half times that extracted by saponification. Thin layer chromatography of the extracts showed, as seen in Table 18, that the effect of the addition of base to the DMSO-protein suspension was manifested entirely in the reduction of the amount of label remaining at the origin

Table 17. Effect of KOH on dimethylsulfoxide extraction of the TCA precipitate

Treatment	Counts/min. extracted
DMSO of TCA precipitate	80, 221
DMSO of TCA precipitate, KOH added	47, 970
Saponification of reaction mixture	18, 376

Table 18. Thin layer chromatography of extracts from three different extractions of cell-free reactions

Spot	R_f	Identity by standards	Treatment of TCA precipitate		
			DMSO	DMSO+KOH	Saponification
			Counts/min. in spot		
1	0.00		451	49	43
2	0.15		41	35	28
3	0.225	Ergosterol	288	292	174
4	0.40		1, 077	1, 216	456
5	0.45	Zymosterol	52	47	31
6	0.525		76	76	35
7	0.625	Lanosterol	47	33	20
8	1.00		118	155	38

of the chromatogram which, in this system, contains the very polar materials. This supports the theory that some other methylated lipids may have been extracted when no base was used. Standards are run on the chromatogram to identify the known yeast sterols ergosterol, zymosterol, and lanosterol. The spot numbered 4 is the component 1 of Turner and Parks (1965), a methylated precursor in ergosterol biosynthesis, which accumulates to a greater extent than ergosterol in these cell-free reactions.

If the theory is correct that the effect of the addition of alkali to the DMSO-protein suspension is to change the pH such that certain methylated lipids become charged and thereby unextractable with petroleum ether, then once this charge acquisition is accomplished, further additions of base should have no further effects on the amount of extractable counts. As seen in Table 19, the theory is supported. It may be further noted in Table 19 that removal of dissolved water from the petroleum ether extracts from non-base treated DMSO-protein suspensions using anhydrous sodium sulfate has the same effect as the addition of base to the suspensions.

As a final test of the procedure, it was desired to compare the amount of digitonin precipitable counts which are extracted by DMSO-KOH and by saponification. Furthermore, a determination of the amount of sterols extracted by each method as determined by the Liebermann-Burchard colorimetric assay was performed to assure

Table 19. Effects of base addition and sodium sulfate treatment on DMSO extraction

Tube #	ml 60% KOH added	Na_2SO_4 treatment of solvent extracts	Counts/min. extracted
1	0	-	35,317
2	0.05	-	20,642
3	0.10	-	21,884
4	0.15	-	21,540
5	0	+	19,110
6	0.05	+	18,660
7	0.10	+	19,934
8	0.15	+	18,332

that the radioactivity extracted was an accurate measure of sterol synthesis. The results are in Table 20. It can be seen that there are both more counts and more Liebermann-Burchard sterols extracted by the DMSO-KOH procedure. However, there is a slight discrepancy in that while the radioactive material extracted by saponification is only 60% of that found by DMSO-KOH, the Liebermann-Burchard sterols from saponification are 84.5% of those found by DMSO-KOH. A final important point in this table is that the percent of counts precipitable from the total extractable counts by digitonin is the same for both methods.

Table 20. Amounts of sterol extracted by saponification and DMSO-KOH treatments as determined by radioactivity, Liebermann-Burchard test, and digitonin precipitation

Extraction procedure	Total extracted counts/min.	μg sterol by L-B	Counts/min. precipitated by digitonin	% counts precipitated by digitonin
Saponification	13, 571	87	9, 572	70. 6
DMSO-KOH	22, 704	103	15, 570	68. 6

DISCUSSION

At the beginning of this work it was anticipated that the best method for approaching the problem of regulation of yeast sterol synthesis was to look for the classical types of control. These were repression of enzyme synthesis (Jacob and Monod, 1961) and feedback inhibition of enzyme function (Monod, Changeux and Jacob, 1963). Since the proposal of the mechanisms for these types of control many variations from the original hypotheses have been found. However, for this work the basic premises as they were first proposed are sufficient.

The original purpose of the work was actually two-fold. It was hoped that a feedback inhibition could be demonstrated, but it was also anticipated that the inhibition might prove to be at the transmethylation step. If this proved to be the case, then by saturating the system with ergosterol one could cause the accumulation of large amounts of the substrate for the transmethylating enzyme. That substrate could then be isolated and its structure determined.

The first series of experiments presented argued very strongly for a feedback inhibition, possibly of more than one enzyme in the pathway as has been shown in the cholesterol system (Gould and Swyryd, 1966). The first experiment, after five hours aeration in the presence of added ergosterol, showed 25% less incorporation

of both methionine and acetate label when compared to the control (Table 1). Two more whole cell experiments (Tables 3 and 5), aerated three hours and two hours respectively in the presence of added sterol, showed only the inhibition of methionine incorporation, but again by 25%. From these early experiments it could be interpreted that the inhibition of the step prior to methylation is the major site of feedback inhibition. Inhibition of the incorporation of acetate into the nonsaponifiable fraction is an additional control point which is only expressed upon long term incubation in the presence of added sterols.

The fact that the cell-free counterparts of these experiments showed less effect, and that the inhibition which they did show was exactly the opposite of the whole cell results, can also be explained to fit this theory. The enzyme prior to transmethylation which is affected in the whole cells may not be active in these preparations. But, because there are still some precursors to transmethylation which have been carried over in the crude preparation, incorporation of the methionine label is seen, and it is the same for all of the enzyme reactions within a single experiment, since all should contain the same amount of endogenous precursors. By the same argument, the enzyme earlier in the pathway which is sensitive to feedback inhibition and is seen in the lowering of acetate incorporation remains sensitive in the cell-free preparations, and the inhibition

is observed.

The results from thin layer chromatography of the sterols made in the cell-free systems upon first examination argued against this theory. As can be seen (Table 7), the acetate label is all in ergosterol, which could not be the case, if the above theory is true. The acetate label would have to be in some nonmethylated precursor. Turner and Parks (1965), have reported that these cell-free preparations do not make ergosterol. Also, all of the methionine label incorporated by these cell-free preparations can be seen by thin layer chromatography to be in halfasterol, not in ergosterol. This, along with other chromatographic evidence which has recently been obtained, suggests that the acetate label is not in fact in ergosterol, but in another component which travels with ergosterol in this system. That component does not precipitate with digitonin. Therefore, whether or not it is sterolic in nature remains questionable.

Two findings created doubt as to the validity of these results. First of all, as mentioned earlier, when duplicate samples were taken there was no correlation between the results received. Secondly, when cells saved from the previous experiments by storage at $4^{o}C$ were analyzed a second time for incorporation of labeled substrates, the results frequently differed from the first analysis. Whether or not there is some breakdown of product or enzymatic activity occurring during storage at $4^{o}C$ has not been examined, but

it seems unlikely that it would change the results to such an extent.

The results obtained using alkaline-pyrogallol saponification cast further doubt on these theories of regulation. The time course analysis performed using this method in no case could be construed to indicate either a feedback inhibition or a repression when the sterols were added by petroleum ether evaporation. Some feedback inhibition might be indicated by the results observed when the sterols were added by complexing in a yeast extract solution. There appeared to be some inhibition of both acetate and methionine label uptake by the added ergosterol. The inhibition was immediate in the case of acetate uptake, and disappeared completely after four hours of aeration in the presence of ergosterol. This is contrary to the theory that the point of inhibition of acetate incorporation is one of secondary control. The methionine inhibition lasted throughout the ten hours of aeration which were observed, but again the problem of noncorrelation of duplicates is seen. One set of samples showed an obvious inhibition, the other did not.

The results obtained from the experiments in which the lyophilized complex was used were also inconclusive. There seemed to be a complete contradiction of results. Comparison of the activities of cell-free extracts from cells aerated normally, in the presence of complex, and in the presence of ergosterol complex gave results which could be interpreted in two ways.

Either the polysaccharide inhibits activity to a small extent and the added ergosterol has no effect, or the small amount of sterol endogenous to the complex is sufficient to completely saturate the enzymes such that the additional sterol in the ergosterol complex has no effect.

When the same experiment was performed with the exception that the resulting extracts were incubated with the same substrates as were the cells from which they were derived, different results were obtained. Aeration and incubation in the presence of complex again inhibited activity to a small extent. Aeration and incubation in the presence of ergosterol complex, however, caused a stimulation of activity.

Contradictory results were again obtained from the third experiment of this series. Addition of ergosterol to the cell-free preparation from the cells grown anaerobically and aerated normally caused a stimulation of incorporation. Anaerobic growth and aeration in the presence of ergosterol followed by incubation of the resulting cell-free extract in the presence and absence of ergosterol resulted in an inhibition of activity by the addition of ergosterol to the cell-free extract.

The conclusion which can best be made from these results is that the different treatments may in some way cause a sensitization or desensitization of the enzyme to the allosteric effect of added

ergosterol. Effects of this nature have been observed before in the case of feedback inhibition. Stadtman (1966) has observed that growing Escherichia coli on different nitrogen sources changes the sensitivity of the glutamine synthetase enzyme to various feedback inhibitors. Perhaps an analogous case exists here. The presence or absence of ergosterol during various stages of the anaerobic-aerobic shift may change the sensitivity of the allosteric enzyme, causing it to be insensitive at some times and sensitive at other times during the period of treatment.

In these time course analyses of cell-free reactions, any effect which is observed, whether it is a stimulation or inhibition, is not observed until the fifth hour of incubation. This could mean that there is an accumulation of some end-products which are synthesized during incubation which affect the activity of the transmethylating enzyme. Another explanation might be that there is a differential deterioration of the enzymes in the different tubes. In either case, this factor argues against the effects seen actually being the result of an end-product inhibition. One would expect such an inhibition to be in effect from the start of incubation.

There is one feature which all of these time course studies with whole cells have in common. There is a shifting out of phase of the fluctuations of label uptake caused by the addition of exogenous sterols. This is especially true of the experiments in which acetate

uptake was observed. Considering this phase shift, one can see that if only a single sample were to be taken during the time of aeration, then depending upon the time at which that sample were taken, different results might be received. For example, in Figure 1, if a sample were taken at any time from one through six hours, analysis would show a stimulation caused by the added sterol. However, if a sample were taken from eight to twelve hours, then the results would show an inhibition of synthesis caused by the added sterol. This type of phenomenon could explain some of the varying results received during the early whole cell experiments. The phase of the fluctuations can be changed by many factors, of which glucose concentration and addition of yeast extract solution are two.

The importance of the time factor in sampling for sterol synthesizing activity cannot be overemphasized. The effect is seen again in the study of the suppression of sterol synthesis in cells grown aerobically in the presence of added sterols. There, it was noted that when cells were grown 40 hours before the preparation of the enzyme, the suppression of activity was 28%. When the preparation was made after 30 hours of growth, the suppression of activity was only 14%. The subsequent study of the relative rates of sterol synthesis during growth in the presence and absence of added sterols showed that these rates also vary with time.(Figure 9). The relative rates of synthesis of sterol become out of phase just

as in the resting cell experiments. The rate for the control peaks earlier during growth than the rates for the samples with added sterols. Thus, there are times when the rates of sterol synthesis are quite different, and others when the rates are almost the same. Different results might therefore be obtained depending upon from which stage of growth the cell-free preparation was made. This point is supported by the experiment shown in Table 11. Cell-free extracts were prepared from cells grown 26 hours under the same conditions as had been used for the whole cell experiment. The same relative activities were seen in the extracts as would have been predicted from the rates of sterol synthesis in the whole cells at that time. If the effect is a feedback inhibition, the sterol acting as the allosteric effector must remain bound to the allosteric enzyme after that enzyme has been extracted. Linn (1965b) has shown that this does occur in cholesterol biosynthesis. He extracted HMG-CoA reductase from rats which had been fed cholesterol. The activity of the enzyme remained suppressed in the cell-free preparations.

The use of the anaerobic-aerobic shift technique as a tool for studying the control of ergosterol biosynthesis may also have been the cause of some of the variations in results. From the results which have been shown it seems that such shifting may distort the regulatory patterns. This can be derived from the fact that the most conclusive and consistent results have come from the studies with aerobic

growing cells. There are two problems inherent in the anaerobic-aerobic shift technique. When the cells are shifted into a new medium such as the aeration medium, they are given a fresh source of glucose. It has already been mentioned that addition of glucose causes changes in the fluctuations of sterol synthesizing activity. Thus, any shift into new medium brings the cell into an entirely new set of conditions, and its response could be altered such that the normal controlling factors are no longer functional. This new set of conditions could also be a function of the concentration of the cells in the aeration suspension. This wild type yeast strain will reach a concentration of approximately 10^8 cells per ml when grown aerobically in a rich medium such as TCA. The concentration of cells in these aeration suspensions is approximately 10^9 cells per ml. This abnormally high cell concentration may also cause abnormal patterns of control in these resting cell suspensions.

A problem which might have been encountered in the cell-free experiments utilizing the lyophilized complex was that of too low a concentration of added sterols. In attempting to add the sterols in the soluble complex, the final concentration may have been too small. At 5 μg ergosterol per ml, there is less than 0.002 μ moles of ergosterol per ml. Such a low concentration may not have been sufficient to cause a feedback inhibition. A normal cell-free reaction at completion of incubation will have approximately 50 μg total of sterol.

In the experiments in which some effect was seen, between 150 and 200 μg of sterol were added per ml. This is in the range of 0.50 to 0.75 μ moles of sterol per ml. In the studies of Jacob and Monod (1961) the concentrations of corepressor used were within this range. Also in feedback inhibition studies this is often the range of concentration of allosteric effector used (Gerhardt and Pardee, 1963; Changeux 1963). The low concentration of sterol added may have therefore been a reason for some of the negative results received.

Another factor which may have influenced these results was permeability. The question can be raised as to whether or not the sterol added to these reactions is actually entering the cell where it can influence synthesis. It is hoped that the use of the soluble complex has alleviated some of this problem. No attempt has been made here to demonstrate the existence of a permease for any of the yeast sterols, and there are no reports in the literature which indicate the existence of a permease. There is some indirect proof that at least some of the added sterols can penetrate the cell membrane. Turner and Parks (1965) showed that when they fed a yeast suspension a methylated precursor to ergosterol which had been labeled with C^{14}, that precursor was at least partially converted to ergosterol. The precursor was added to the flasks by the method of petroleum ether evaporation. By feeding a suspension of yeast ergosterol-C^{14}, then harvesting the yeast and washing them several

times, one can also recover upon saponification and extraction some of the ergosterol-C^{14}. However, it cannot be proved that the ergosterol has not only absorbed to the cell's surface.

In attempting to hypothesize the regulatory factors associated with ergosterol biosynthesis, the results shown here dictate that one must not only consider a direct end-product control. A consideration of the physiological role of ergosterol must also be considered. End-product control of ergosterol biosynthesis, under conditions where it has been observed, has only been to the extent of 25% inhibition of activity. This was at the highest concentration of added sterol. The interpretation could be that there is in reality no end-product control and that the difference is an artifact. Another possible interpretation is that there is a masking effect such that full expression of control is not seen. A third possibility is that for physiological reasons 25% inhibition is maximal for this system.

The work with aerobically growing cells provides good evidence that there is an end-product modulated control, and that it is a feedback inhibition. In these growing aerobic cells the inhibition by ergosterol was seen in both the cell-free preparations and the whole cells, if the cells were not removed from the growth medium. However, when the whole cell experiments were performed in exactly the same manner with the exception that analysis of sterol synthesis was made by suspending the cells in aeration medium, rather than leaving them in the growth medium, then no depression of sterol synthesis

was seen in the ergosterol grown cells. If the control mechanism is a repression of enzyme synthesis, then such a phenomenon should not occur. The cells in the aeration medium are in a resting phase where there is essentially no protein synthesis. There can therefore be no synthesis of new enzyme and no recovery from a depressed level of enzyme as in the case of repression. The results therefore indicate a feedback inhibition as the method of control. This is compatible with the results from the growing cells. The differential rates of synthesis could either be the result of a lowering of the amount of enzyme or of an inhibition of enzyme activity.

There is a discrepancy in the argument for a feedback inhibition of sterol synthesis. The allosteric effector is tightly bound to the enzyme extracted from the cells grown aerobically in the presence of ergosterol. However, addition of ergosterol to normal cell-free preparations does not cause a marked inhibition. Cells grown aerobically with added ergosterol, then transferred to aeration medium, do not show any inhibition of sterol synthesis during the resting phase. The allosteric effector is apparently removed immediately from the enzyme. These results suggest that a third factor may be necessary to mediate the binding of ergosterol to the enzyme. Its subsequent release may also be under such control. In the cell-free extracts the substance is not present, and ergosterol is neither bound nor released from the allosteric enzyme. Thus, there may be

some control over this system from another source. The involvement of ergosterol in the respiratory activity of the cell and in exerting some control over the levels of some of the dehydrogenase enzymes of the cell may provide a hint as to the source of a secondary control.

Ergosterol is necessary for the formation of new cells, if for nothing else than their structural continuity. Thus its rapid synthesis during aerobic growth can be understood. However, ergosterol also seems to maintain some type of check on the metabolism of the cell. Namely, it seems to exert some control over the fermentative versus oxidative processes in the cell. This is supported by the work of Tauson (1948), Matkovics and Konschansky (1958), and Deborin, et al. (1960), who have shown that ergosterol causes an increase in the fermentative activity of the cell and a lowering of the oxidation-reduction potential of the cell. As a result of this activity, the control over ergosterol biosynthesis may reside in a form of catabolite repression. Its rate of synthesis increases rapidly during early growth of the cell, and with that increased rate the ergosterol will influence the type of metabolism of the cell. Thus, the breakdown of glucose should be fermentative, producing primarily ethanol and glycerol. This is so under these conditions. Then, perhaps the accumulation of these catabolites or some related compounds causes an allosteric inhibition of the sterol

synthesizing enzymes, lowering the rate of sterol synthesis in the late log and early stationary phases of growth. As these catabolites are then slowly used in aerobic respiration, ergosterol synthesis again increases in the late stationary phase. This is the pattern of rates of sterol synthesis seen in Figure 9. These catabolites may be associated with a third component involved in binding ergosterol to the allosteric enzyme, as hypothesized earlier.

Thus, it is predicted here that the control of ergosterol synthesis is actually a two phase system. There is a classical feedback control which serves as a partial control over the synthesis, but only to the extent of 25%. Then, there is a type of catabolite repression, in which catabolites resulting from the influence of ergosterol on carbohydrate metabolism reach a critical concentration, at which point they too cause an inhibition of ergosterol synthesis.

This control is exerted only under aerobic conditions. Under anaerobic conditions the problem is nonexistent. There is no aerobic respiration, and ergosterol is not made. There may be some control over the amount of squalene made, and this could be under a catabolite repressive type of control.

Under the artificial conditions imposed upon the system by the anaerobic-aerobic shift technique, another mechanism for regulation might exist. Klein (1955), as stated earlier, has found that

under these conditions glucose will be used for sterol synthesis before the squalene in the pool which had accumulated during anaerobic growth. This argues for the existence of two squalene pools, one made anaerobically and one aerobically. This would also keep the entire synthetic process, not just that part from squalene to ergosterol, under the aerobic controls under these conditions. However, the lack of any demonstrable end-product control in resting cells argues that this system differs in still another way from that of growing aerobic cells.

The specific steps of sterol synthesis involved in this catabolite and end-product control cannot be hypothesized at this time.

The discovery of the inhibitory capacity of yeast extract on the methyl-C^{14} uptake of the cell-free sterol synthesizing system from yeast at first led to speculation that there was some water soluble substance, such as a catabolite, which was the controlling factor for the regulation of sterol synthesis. However, ashing of the inhibitor containing solution proved that the inhibitor was inorganic in nature. Treatment of the inhibitor solution with a cation exchange resin then demonstrated the cationic nature of the inhibitor.

The results also suggest that the inhibition is not caused by only one factor. This evidence is presented in Table 13, where it can be seen that a solution of standard potassium phosphate of the same concentration as that determined for the inhibitor solution

causes a small amount of inhibition (10%). Also, in Table 14 Dowex treatment of the inhibitor solution removed only 80% of the inhibitory activity of the control solution. Thus, the evidence suggests that the total inhibitory capacity of the yeast extract solution is a result of several different components. The major contributor is the cationic fraction. Whether or not all of the inhibition by the cationic fraction is caused by one particular cation has not been determined.

The importance of this inhibitory ion is that it differs in its function from others which have been studied (Turner, Larson, and Parks, 1965; Parks and Starr, 1963). Copper sulfate was the only salt studied by these groups which gave the large inhibition of transmethylation. The comparison of the inhibitory activities of copper sulfate and the yeast extract inhibitor in Figure 10 demonstrates the difference in the action of the two substances. In Figure 10 (c) it can be seen that the yeast extract inhibitor lowers the amount of counts incorporated per μg of sterol. Copper sulfate does not do this. This means that copper sulfate lowers both the amount of total sterol and the amount of label incorporated. The yeast extract inhibitor lowers only the amount of methyl-C^{14} label incorporated, and not the amount of total sterol. It is implied from these results that copper sulfate lowers total sterol synthesis, while the yeast extract inhibitor stops only transmethylation, allowing the buildup of precursors to transmethylation which are Liebermann-Burchard

positive. It is hoped that the inhibitor can be used to cause the accumulation of large amounts of the substrate for transmethylation. This compound could then be isolated.

It should be noted that the inhibition is much greater when methionine is used as the source of methyl groups than when S-adenosylmethionine is used as that source. Part of the observed inhibition may therefore be at the AM synthetase step, the enzymatic synthesis of AM from methionine and ATP. Even if this is the case, in the cell-free reactions the effect of inhibition should remain the same. It is doubtful that the amount of endogenous AM in the extracts is large enough such that it could convert all of the substrate for transmethylation to a methylated intermediate.

From the study of extraction procedures for cell-free reactions it is evident that DMSO is much more efficient than saponification in releasing the sterols for extraction in cell-free extracts of yeast. The method has also proved to give much more consistent yields than saponification within any single experiment. From the work here and from that of Adams and Parks, it can be predicted that this greater efficiency is a result of the DMSO having the ability to release the sterol from both the protein and the water soluble complex.

It has been found in other work that the amount of digitonin precipitable counts obtained from extracts of DMSO suspensions

treated with and without base are the same. This indicates that the extra counts extracted when no base is used are most likely contained in nonsterolic constituents as the thin layer chromatography results also indicate. No attempt has been made to identify these constituents. The fact that drying performs the same function as base addition may be due to charge effects of the salt attracting these more polar lipids from the nonpolar environment of the petroleum ether.

Only about 70% of the counts extracted by either DMSO-KOH or saponification of these cell-free reactions are precipitable by digitonin. Adams and Parks (1967b) have shown that in whole cells of yeast 90% of the nonsaponifiable counts are digitonin precipitable. The basis for this discrepancy between the whole cell and cell-free experiments is not obvious. However, in the cell-free system some components which are nondigitonin precipitable may be synthesized which are not similarly accumulated in the whole cell. The methylated ergosterol precursors found in in vitro experiments but only under very limited conditions in vivo are examples (Turner and Parks, 1965). Alternatively, some constituents may be used in such a manner in the whole cell that they are not extracted by these methods. In addition, digitonin precipitation is only a measure of 3-β-hydroxy sterols, and therefore nonprecipitable components cannot necessarily be termed nonsterolic.

SUMMARY

The regulation of ergosterol biosynthesis in yeast has been studied. The system has been examined for the presence of an end-product inhibition. Both anaerobically and aerobically grown cells have been examined for this effect. A feedback inhibition of sterol synthesis has been shown, but it appears to be a secondary effect. The regulation is intimately involved with the carbohydrate metabolism of the aerobic cell. Other involvements may exist in anaerobic cells. A type of catabolite repression of ergosterol biosynthesis has been hypothesized.

An inhibitor of sterol synthesis which is cationic in nature has been found in yeast extract. The inhibitory effect is directed specifically at transmethylation, and may cause the accumulation of precursors to transmethylation in ergosterol synthesis.

A procedure has been developed for extracting sterols from cell-free extracts of yeast. The method gives larger and more consistent yields of sterol than the conventional methods of extraction.

BIBLIOGRAPHY

Adams, B. G. and L. W. Parks. 1967a. A water-soluble form of ergosterol and cholesterol for physiological studies. Biochemical and Biophysical Research Communications 28:490-494.

Adams, B. G. and L. W. Parks. 1967b. Evidence for dual physiological forms of ergosterol in Saccharomyces cerevisiae. Journal of Cellular Physiology 70:161-168.

Adams, B. G. and L. W. Parks. 1968. Isolation from yeast of a metabolically active water-soluble form of ergosterol. Journal of Lipid Research 9:8-11.

Akhtar, M., P. F. Hunt and M. A. Parvez. 1966. Mechanism of the alkylation step in ergosterol biosynthesis. Chemical Communications, 1966, p. 565-566.

Akhtar, M., M. A. Parvez and P. F. Hunt. 1966. The synthesis of labeled 24-methylenelanosterol and its conversion into ergosterol. Biochemical Journal 100:38c-40c.

Alexander, G. J., A. M. Gold and E. Schwenk. 1957. The methyl group of methionine as a source of carbon 28 in ergosterol. Journal of the American Chemical Society 79:2967.

Andreason, A. A. and S. J. B. Stier. 1953. Anaerobic nutrition of Saccharomyces cerevisiae. I. Ergosterol requirement for growth in a defined medium. Journal of Cellular and Comparative Physiology 41:23-36.

Andreason, A. A. and S. J. B. Stier. 1954. Anaerobic nutrition of Saccharomyces cerevisiae. II. Unsaturated fatty acid requirement for growth in a defined medium. Journal of Cellular and Comparative Physiology 43:271-281.

Barton, D. H. R., D. M. Harrison and G. P. Moss. 1966. 24-methylenedihydrolanosterol as a precursor of steroids and triterpenoids. Chemical Communications, 1966, p. 595-596.

Breivic, O. N., J. L. Owades and R. F. Light. 1954. A new tetraethenoid sterol of yeast. Journal of Organic Chemistry 19:1734-1740.

Changeux, Jean-Pierre. 1963. Allosteric interactions on biosynthetic L-threonine deaminase from E. coli K-12. Cold Spring Harbor Symposia on Quantitative Biology 28:497-504.

Chuang, C. K. 1933. Über die Konstitution des Ergosterins. Justus Liebigs Annalen der Chemie 500:270-280.

Clayton, R. B. and Konrad Bloch. 1956a. Biological synthesis of lanosterol and agnosterol. Journal of Biological Chemistry 218:305-318.

Clayton, R. B. and Konrad Bloch. 1956b. The biological conversion of lanosterol to cholesterol. Journal of Biological Chemistry 218:319-325.

Cornforth, J. W. 1959. Biosynthesis of fatty acids and cholesterol considered as chemical processes. Journal of Lipid Research 1:3-28.

Danielson, H. and Konrad Bloch. 1957. Origin of carbon atom number 28 in ergosterol. Journal of the American Chemical Society 79:500-501.

Dauben, W. G., T. W. Hutton and G. A. Boswell. 1959. Biosynthesis of ergosterol: its relation to the squalene hypothesis. Journal of the American Chemical Society 81:403-407.

Deborin, G. A. et al. 1960. Influence of ergosterol on the enzymic action of phosphoglyceraldehyde dehydrogenase. Acta Physiologica Academiae Scientiarum Hungaricae 17:133-140. (Abstracted in Chemical Abstracts 54:21225f. 1960)

Eaton, N. R. 1962. A new press for the disruption of microorganisms. Journal of Bacteriology 83:1359-1360.

Fieser, Louis F. and Mary Fieser. 1959. Steroids. New York, Reinhold. 945 p.

Fisk, C. H. and Y. Subbarow. 1925. A colorimetric determination of phosphorus. Journal of Biological Chemistry 66:375-400.

Gerhart, J. C. and A. B. Pardee. 1963. The effect of the feedback inhibitor, CTP, on subunit interactions in aspartate transcarbamylase. Cold Spring Harbor Symposia on Quantitative

Biology 28:491-496.

Gould, R. Gordon and E. A. Swyryd. 1966. Sites of control of hepatic cholesterol biosynthesis. Journal of Lipid Research 7:698-707.

Hanahan, D. J. and S. J. Wakil. 1953. Origin of some of the carbon atoms of the side chain of C^{14} ergosterol. Journal of the American Chemical Society 75:273-275.

Issidorides, C. H., I. Kitagawa and E. Mosettig. 1962. Cleavage of steroidal digitonides in dimethyl sulfoxide. Journal of Organic Chemistry 27:4693-4694.

Jacob, F. and J. Monod. 1961. Genetic regulatory mechanisms in the synthesis of proteins. Journal of Molecular Biology 3:318-356.

Katsuki, H. and Konrad Bloch. 1967. The biosynthesis of ergosterol in yeast: formation of methylated intermediates. Journal of Biological Chemistry 242:222-227.

Kingdon, H. S. and E. R. Stadtman. 1967. Two E. coli glutamine synthetases with different sensitivities to feedback effectors. Biochemical and Biophysical Research Communications 27:470-473.

Klein, H. P. 1955. Synthesis of lipids in resting cells of Saccharomyces cerevisiae. Journal of Bacteriology 69:620-627.

Klein, H. P. 1957. Some observations on a cell free lipide synthesizing system from Saccharomyces cerevisiae. Journal of Bacteriology 73:530-537.

Kodicek, E. and P. R. Ashby. 1957. Formation of carbon-14 labeled sterols by yeast. Biochemical Journal 66:35p-36p.

Krámli, A. and J. Lantos. 1956. Action of oxidation-reduction systems on the metabolism of microorganisms. III. Effect of thioglycolic acid on ergosterol production in yeast cultures. Acta Biologica Academiae Scientiarum Hungaricae 6:185-191. (Abstracted in Chemical Abstracts 50:15698i. 1956)

Linn, Tracy C. 1967a. The demonstration and solubilization of β-hydroxy-β-methylglutaryl coenzyme A reductase from rat liver microsomes. Journal of Biological Chemistry 242: 984-989.

Linn, Tracy C. 1967b. The effect of cholesterol feeding and fasting upon β-hydroxy-β-methylglutaryl coenzyme A reductase. Journal of Biological Chemistry 242: 990-993.

Lowry, O. H. et al. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265-275.

Maguigan, W. H. and Ernst Walker. 1940. Sterol metabolism of microorganisms. I. Yeast. Biochemical Journal 34: 804-813.

Matkovics, B. 1958. Über die Wirkung von Sterinen auf Mikroorganismen. III. Die Wirkung des Ergosterins auf das Redoxpotential und den Trockensubstanzgehalt von Penicillin chrysogenum Q 176 Kulturen. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. Abteil 2, 111: 543-545.

Monod, Jacques, Jean-Pierre Changeux and F. Jacob. 1963. Allosteric proteins and cellular control systems. Journal of Molecular Biology 6: 306-329.

Morpurgo, G. et al. 1964. Influence of ergosterol on the physiology and ultrastructure of Saccharomyces cerevisiae. Nature 210: 897-899.

Nightingale, M. S., Su-Chen Tsai and J. L. Gaylor. 1967. Testicular sterols. VI. Incorporation of mevalonate into squalene and sterols by cell-free preparations of testicular tissue. Journal of Biological Chemistry 242: 341-349.

Ogilvie, J. W. and B. H. Kaplan . 1966. The inhibition of sterol biosynthesis in rat liver homogenates by bile. Journal of Biological Chemistry 241: 4722-4730.

Olson, J. A., M. Lindberg and Konrad Bloch. 1957. On the demethylation of lanosterol to cholesterol. Journal of Biological Chemistry 226: 941-956.

Parks, L. W. 1958. S-adenosylmethionine and ergosterol synthesis. Journal of the American Chemical Society 80: 2023-2024.

Parks, L. W. and P. R. Starr. 1963. A relation between ergosterol and respiratory competency in yeast. Journal of Cellular and Comparative Physiology 61:61-65.

Parks, L. W., J. R. Turner and R. L. Larson. 1965. Transmethylation in yeast sterol synthesis. In: Transmethylation and methionine biosynthesis, ed. by Stanley K. Shapiro and Fritz Schlenk. Chicago, University of Chicago. p. 85-93.

Ponsinet, Gerard and Guy Ourisson. 1965. Yeast methylsterols. Bulletin de la Société Chimique de France, 1965, p. 3682-3684. (Abstracted in Chemical Abstracts 64:11277f. 1966)

Rosenheim, O. and T. A. Webster. 1927. The parent substance of vitamin D. Biochemical Journal 21:389-397.

Schwenk, E. and G. J. Alexander. 1958. Biogenesis of yeast sterols. II. Formation of ergosterol in yeast homogenates. Archives of Biochemistry and Biophysics 76:65-74.

Siperstein, M. D. and J. M. Guest. 1960. Feedback control of cholesterol synthesis. Journal of Clinical Investigation 39: 642-652.

Smedley-Maclean, I. and E. M. Thomas. 1920. The nature of yeast fat. Biochemical Journal 14:483-493.

Tanret, C. 1889. Sur on nouveneau principe immediate de l'ergot de seigle, l'ergosterine. Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences 108:98. (Cited in: Fieser, Louis F. and Mary Fieser. Steroids. New York, Reinhold, 1959. p. 94)

Tauson, T. A. 1948. Influence of sterols on activity of microorganisms. I. Physiological effects of ergosterol on yeasts and endomyces. Microbiologiia 17:127-131. (Abstracted in Chemical Abstracts 44:4078a. 1950)

Tchen, T. T. and Konrad Bloch. 1957. On the mechanism of enzymatic cyclization of squalene. Journal of Biological Chemistry 226:931-939.

Tomkins, G. M., H. Sheppard and I. L. Chaikoff. 1953. Cholesterol synthesis by liver. III. Its regulation by injested cholesterol. Journal of Biological Chemistry 201:137-141.

Turner, J. R. and L. W. Parks. 1965. Transmethylation products as intermediates in ergosterol biosynthesis in yeast. Biochimica et Biophysica Acta 98:394-401.