AN ABSTRACT OF THE THESIS OF

BRUCE GORDON ADAMS for the Ph. D.
(Name of student) (Degree)

in Microbiology (Microbial Physiology) presented on April 25, 1968
(Major) (Date)

Title: A STUDY OF THE FUNCTION AND PHYSIOLOGICAL FORMS OF ERGOSTEROL IN SACCHAROMYCES CEREVISIAE

Abstract approved: [redacted]
Leo W. Parks

A water-soluble complex containing ergosterol together with a component of yeast has been isolated. The complex can be isolated from commercial yeast extract to which ergosterol has been added or directly from whole yeast cells. The complexing agent from yeast extract is also capable of solubilizing cholesterol and a long chain hydrocarbon, hexadecane. The complexing agent has been shown to be a polysaccharide and appears to be composed solely of glucose subunits. The complexing agent does not appear to be glycogen. The binding between the sterol and the polysaccharide appears to be noncovalent. The complex is easily prepared and is stable in aqueous solution; ergosterol in this solution is metabolically available to yeast cells to which it is added.

Data obtained from acid hydrolysis and extraction of yeast have demonstrated that routine saponification does not recover total

sterol from the cells. This suggests the existence of a form of ergosterol resistant to saponification. Time course analyses of sterol synthesis by nonproliferating cell suspensions reveal an inverse relationship between the amounts of base labile and acid labile forms of sterol. These data give strong presumptive evidence for dual forms of ergosterol which are interconvertible according to the respiratory state of the cell.

Experiments dealing with the effect of respiratory inhibitors on sterol synthesis in nonprofilerating cell suspensions suggest that the synthesis and physiological form of ergosterol is intimately related to the integrity of the respiratory apparatus and that the DNA encoding for the synthesis and regulation of ergosterol is located in the mitochondria.

A Study of the Function and Physiological Forms of Ergosterol in *Saccharomyces cerevisiae*

by

Bruce Gordon Adams

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1968

ACKNOWLEDGEMENT

To Dr. Leo W. Parks:

.... for his guidance, his inspiration, and his confidence in the course of this investigation.

To my wife, Lynn:

.... for her infinite patience, her encouragement, and her uncompromised support during the past three years.

To my parents:

.... for their continued encouragement and support throughout the years.

To the staff and students of Oregon State University:

.... for their advice and hospitality while I have been at Oregon State.

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Date thesis is presented April 25, 1968

Typed by Velda D. Mullins for Bruce Gordon Adams

This thesis is dedicated to the green hills of Africa.

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INTRODUCTION

Although the synthesis of cholesterol and its subsequent conversion to the steroid hormones has been well documented, the precise role of ergosterol and other yeast sterols has as yet not been elucidated. The study of ergosterol in yeast has been the subject of rather intensive investigation for a number of years. The sterol is known to compose up to 10% of the dry weight of cells in yeast and thus cannot be considered a minor constituent. Although many roles for ergosterol have been proposed, there have been and still remain inconsistencies and disagreement on both the methods of investigation used in the study of ergosterol and on the results and conclusions obtained with these methods.

A relationship between ergosterol synthesis and the respiratory state of the yeast cell has been recognized for some time. However, definitive experiments concerning the exact nature of this relationship have been lacking. By considering the substantial data accumulated from studies on respiration deficient strains of yeast and what is known concerning the relationship of ergosterol synthesis to respiration, an attempt has been made to define more

precisely the relationship between ergosterol and ergosterol derivative levels of yeast cells and the physiological state of the cell as a whole. It is hoped that some of the observations made during these studies will eventually aid in a precise definition of the role of ergosterol in yeast.

REVIEW OF LITERATURE

Ergosterol was first isolated from ergot of rye in 1889 by Tanret (90). An impure form of the sterol was isolated from yeast by Gerard (29), and subsequently Smedley-MacLean isolated a purified form of ergosterol from Saccharomyces (75). Although ergosterol comprises 80-85% of the total sterol under normal conditions, yeast are known to contain many other sterols; among them are zymosterol, ascosterol, fecosterol, episterol, 5-dihydroergosterol, 14-dehydroergosterol, hyposterol, cerevisterol, ergostatetraenol, and lanosterol (26, p. 355-358).

The complete structure of ergosterol was established in 1933 (26, p. 108) and is shown in Figure 1, along with the structure of cholesterol. Ergosterol has a conjugated double bond system in the B ring at positions 5-6 and 7-8, a β-hydroxyl group at position 3 and another double bond system in the B ring at positions 5-6 and 7-8, and another double bond in the side chain at position 22-23. Ergosterol gives a fast reacting positive Liebermann-Burchard reaction and forms an insoluble complex with digitonin. The sterol is extremely insoluble in water but is soluble in ethanol, benzene, ether, chloroform and most other fat solvents. Ultraviolet light is strongly

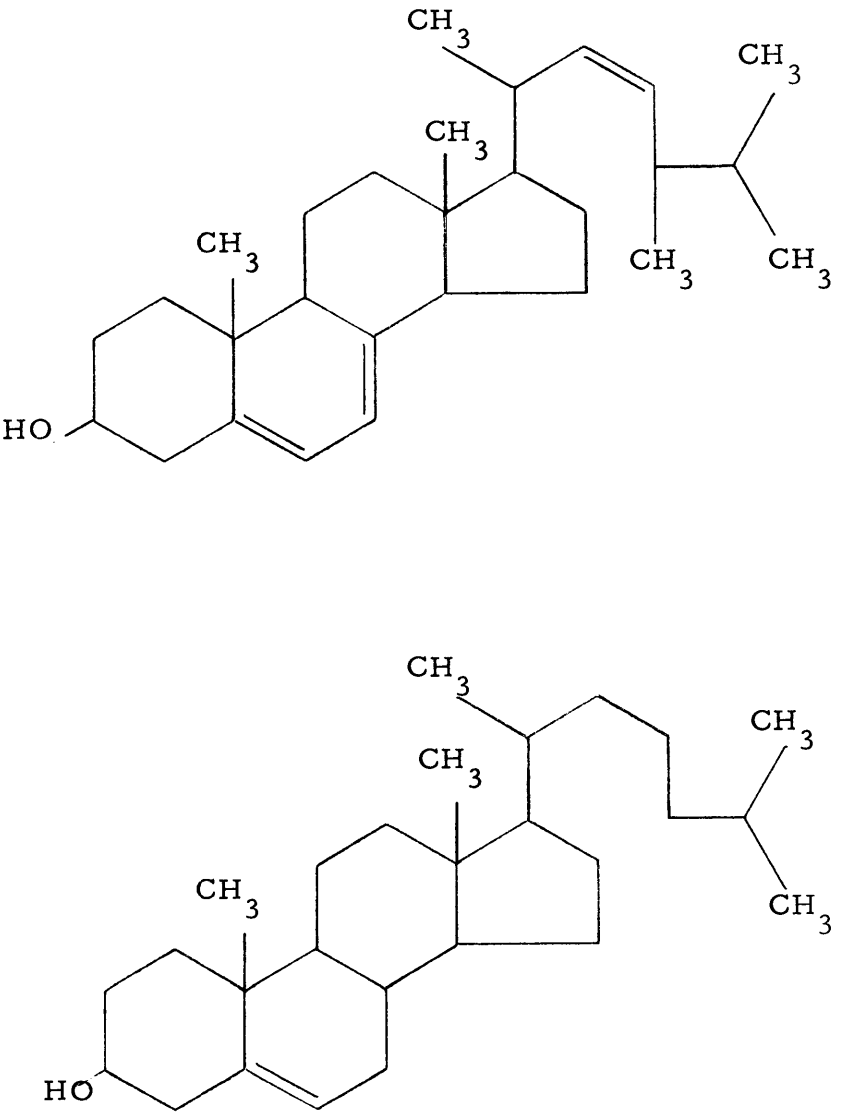


Figure 1. The structural formula for ergosterol is shown in the upper figure; the structural formula for cholesterol is shown in the lower figure.

absorbed by ergosterol, and in ethanol solution gives absorption peaks at 271 mμ, 282 mμ, 294 mμ, and a shoulder at 262 mμ (26 p. 93).

Other than the additional unsaturation in the B ring and at position 22-23 in the side chain, ergosterol differs from cholesterol in possessing an additional methyl group attached at the 24th position. The origin of this methyl group has been of interest because it arose independently of the remainder of the sterol molecule. Danielson and Bloch (15) observed that this methyl group could arise from a variety of one carbon sources and Parks (63) succeeded in demonstrating that S-adenosylmethionine was preferentially used as the methyl source for sterol synthesis in yeast.

Ergosterol is apparently synthesized by all yeast. Dulaney and co-workers (20) studied the ergosterol production of 558 yeast cultures comprising 69 species in 20 genera and observed a range of from 0.1% to 10.7% dry weight of ergosterol. The sterol content of all genera except Saccharomyces was about 0.1%, with some cultures of Saccharomyces cerevisiae producing 7-10% dry weight of ergosterol. The authors noted that the concentration of ergosterol in the strains varied considerably depending upon the strain and the growth conditions. The strains of Saccharomyces cerevisiae averaged 2% to 5% of ergosterol.

Although yeast contain by far the largest amounts of sterol,

other simple forms of life have been reported to contain sterol. Sterols have been claimed to have been isolated from saprophytic protozoa (101, 86), from parasitic protozoa (30, 31) and from molds (68). The existence of sterols in bacteria has been and still remains a controversy. Claims have been made for the detection of ergosterol in Escherichia coli, Lactobacillus arabinosus, Lactobacillus pentosus, and Azotobacter chroococum (25). A component isolated from the nonsaponifiable fraction of Micromonospora species behaved with digitonin and on columns like ergosterol and was calculated to comprise 0.001% of the wet weight of the organism. Similar assays on Sphaerotilus natans and Streptomyces griseus yielded negative results in tests that could detect as little as 0.00001% wet weight as sterol (25). Aaronson (1) has proposed that sterols play a role in the metabolism of the purple photosynthetic bacterium Rhodopseudomonas palustris because the hypocholesteraemic agents benzmalcene and triparonal inhibit the growth of this organism. He found that this inhibition could be overcome by the addition of oleic acid, ergosterol, squalene, farnesol or lanosterol, but not by mevalonic acid, geranyl acetate nor palmitic and stearic acids.

It has long been known that the amount of ergosterol formed by yeast is a function of carbohydrate metabolism. Massengale and co-workers (51) noted that the principal determinant of ergosterol production was the carbon source and showed that there was no clear

correlation of the sterol content with total fat content or nitrogen content in growing cultures. They found the most efficient carbon source to be glucose, followed by fructose, mannose and galactose. In an extensive survey, Maguigan and Walker (50) observed a number of features concerning the synthesis of sterols in yeast. Using non-proliferating suspensions of commercial baker's yeast, they showed that sterol production was a feature of aerobic metabolism, that the synthesis of sterols arose early in the course of aeration and that it was accompanied by an increase in total lipid. The sterol was formed at a much greater rate than the slight growth occurring in their cultures, and it was found that most of the sterol was esterified. They also showed that the addition of casein hydrolysate to the aeration medium resulted in a 50% reduction in the sterol content of the cells. Sucrose was found to be the most efficient carbon source, and they noted that although sodium acetate by itself gave but one-third the amount of total sterol as did sucrose, the addition of acetate to the sugar containing medium resulted in a 20% increase in sterol production over that of sugar alone. They concluded from this that acetate could play the role of a carbon source in sterol synthesis in yeast.

It was subsequently shown that acetate can serve as the major carbon source for both ergosterol and cholestrol (13). It has been demonstrated that the coenzyme A levels of the cell are directly proportional to ergosterol synthesis (39), and that if coenzyme A is

removed from cell free sterol synthesizing systems by ion exchange adsorption, no sterol synthesis occurs until the cofactor is replaced (11). In addition, Rajagopalan and Sarma (68) reported a stimulation of sterol synthesis, when yeast were grown in the presence of sulfanilamide. They showed that increasing levels of the drug resulted in increasing levels of coenzyme A, increasing levels of both free and total sterol, and a decreasing yield of cell growth. They concluded that the enhancement of the coenzyme A levels represented a metabolic response to the drug, the increase in sterol being a direct consequence of the increased coenzyme A levels. Starr and Parks (85), however, demonstrated a two-fold decrease in sterol content of cells grown in the presence of even small amounts of sulfanilamide and that this decrease in sterol resulted in no significant alteration in the cell yield. They also showed that aerated nonproliferating cells in the presence of the drug exhibited no significant alteration in sterol content. They concluded that for inhibition of sterol synthesis to occur, the drug must be incorporated into some cellular unit during growth.

Gal'tsova and co-workers (27) showed that ergosterol synthesis is favored by excess sugar and nitrogen deficiency; the addition of ammonium sulfate, casein hydrolysate and glycine resulted in a decrease in sterol content of 14%, 20%, and 15 to 20%, respectively. Starr and Parks (85) demonstrated that in aerated nonproliferating

yeast suspensions only glucose and ethanol were available for sterol synthesis; acetate, succinate and glycerol gave values below a control containing no carbon source. In growing cultures they noted that although growth in media utilizing glycerol, acetate, xylose and succinate was very poor compared to that obtained with glucose, the amount of sterol per mg dry weight cells was much greater. They showed that these carbon sources and media poor in nitrogenous intermediates yielded cells with the greatest sterol content. Thus there exists an inverse relationship between sterol content and cell yield. The authors postulate that carbon intermediates may be shunted towards the formation of products other than sterols, when more nitrogenous intermediates are provided.

The biosynthetic reactions leading to the synthesis of mevalonic acid, squalene and lanosterol, intermediates common to both ergosterol and cholesterol, have been found to follow parallel courses in both yeast and mammalian systems (14). Enzymes catalyzing identical reactions leading to lanosterol have been found in cell-free preparations from both systems. Although the pathway leading from lanosterol to cholesterol has been extensively studied, the pathway to ergosterol is considerably less well understood. Using cell-free preparations of yeast, Turner and Parks (97) identified the methylated nonsaponificable products obtained from transmethylation from S-adenosylmethionine as sterols. At least three sterols possessing the

transferred methyl group were detected; one of these, detected in by far the largest amounts, is ergosterol. One of the two unidentified methylated sterols, present in transitory amounts, was demonstrated to be a precursor of ergosterol, methylation being apparently the final stage of synthesis of ergosterol.

Maguigan and Walker (50) demonstrated that ergosterol production was a product of aerobic metabolism. It was shown that ergosterol synthesis is a function of oxygen utilization not related to, or dependent on, cell growth. Simultaneous determination of ergosterol, fermenting capacity and oxygen consumption revealed that the formation of ergosterol coincided with the oxidative activity of the cell. Klein (37)noted that anaerobic yeast synthesize little, if any, ergosterol, when grown in the absence of oxygen and that the resulting population vigorously aerated with a suitable carbon source gave rise to a three to eight-fold increase in sterol production in 24 hours. In support of these observations, Tchenn and Bloch (93) demonstrated that under anaerobic conditions squalene is accumulated intracellularly and that molecular oxygen is necessary for the subsequent formation of ergosterol, being required as a source of the 3-β-hydroxy group, for the removal of the extra methyl groups of lanosterol, and for the shifting of the double bonds in the late states of sterol synthesis.

The exact site of sterol synthesis within the cell is as yet

unknown, Klein and Booher (36) reported that both the particulate and supernatant fractions of cell free extracts of yeast are required for the synthesis of ergosterol. The particulate fraction was suspected to be limiting since an increase in the amount of particulate fraction increased the sterol content. Subsequently, however, Klein and Greenfield (38) have demonstrated that the enzymatic apparatus for lipid synthesis is exclusive of the mitochondrion. They conclude that the mitochondrion may be involved in activation and control of lipogenisis in intact cells rather than in the actual synthesis.

Johnston (34) has shown that streptomycin added to both whole cell and cell free sterol synthesizing systems inhibits the synthesis of sterols. Stewart and Wooley (87) observed that the addition of the mevalonic acid analogue 3-hydroxy-3-methyl-5-phenyl-4-pentenoic acid at a concentration causing a reduction in sterol synthesis did not cause an inhibition of growth. Higher levels of the analogue did not increase the inhibition of synthesis even at growth inhibiting concentrations.

Ergosterol has been reported to exist in yeast in a variety of forms. Maguigan and Walker (50) reported that ergosterol in yeast may exist either in the free form or esterified with fatty acids at the 3-hydroxyl group, with most of the sterol existing in the latter form. However, recent investigations on the esters of ergosterol

by gas liquid chromatography have shown a maximum sterol ester content of 14% of the total sterol (49).

There have been numerous reports of sterol being bound to other cellular constituents. Nyman and Chargaff (61) have described the isolation of a lipoprotein particulate fraction which contained 25% lipid of which 33% was ergosterol. Vendt (99) has evidence suggesting that ergosterol may be linked to an indole or imidazole group or protein through the 5-6 double bond. Trufanov and Kirsanova (96) report that ergosterol is released into the medium in a soluble, combined form upon the autolysis of yeast. Additionally, Deborin and Gorbacheva (17) have demonstrated that globules of egg albumin absorb varying quantities of ergosterol. They find that a protein-sterol complex is formed at 37°C to 45°C which consists of one mole of ergosterol to two moles of albumin. This complex does not undergo spontaneous denaturation and the authors suggest this complex to be similar to native protein-sterol complexes. In a subsequent communication Deborin and Shibanova (18) show that ergosterol absorbed by egg albumin causes a doubling of the molecular weight of the albumin giving a dimer which retains a shape similar to that of the original molecule.

Investigators have used a variety of methods for suspending sterols in aqueous media. The methods range from simply adding the sterol as crystals (92), adding the sterol in ethanol (69, 56),

evaporating the sterol in solvent on the sides of the flask (97) to suspension in albumin (64), lecithin (24), agar (2), sodium deoxycholate (2) or propylene glycol (24). By far the most prevalent method of suspension has been with the use of Tween-80 (2, 24, 64, 41, 98, 47, 84). Tween-80 is polyoxyethylene sorbitan monoleate and contains approximately 0.6% unesterified oleic acid (69).

Although the synthesis of cholesterol and its subsequent conversion to the steroid hormones has been well documented, the precise role of sterols in yeast has as yet not been elucidated. In efforts to define the role of ergosterol in Saccharomyces cerevisiae, several investigators have sought to isolate mutants of yeast which cannot synthesize ergosterol and which would require the addition of exogenous sterol for growth. Adelberg and co-workers (2) used ultraviolet irradiation as a mutagen for cultures of Saccharomyces cerevisiae. Of 20,267 surviving individual cultures, 1,817 postenrichment colonies were picked and 218 of these exhibited a temporary requirement for ergosterol. Unfortunately no permanently deficient strains were isolated. They found the temporary mutants to grow rapidly in the presence of ergosterol when first isolated and slowly or not at all in its absence. However, in only one instance did the deficiency persist through the second subculture of the mutant strains and none persisted through the third. Recently Resnick and Mortimer (69) reported the isolation of

several strains of Saccharomyces cerevisiae which exhibited a requirement for ergosterol. This requirement, however, could be satisfied by the addition of either oleic acid or ergosterol. These isolates were respiratory deficient and grew poorly even in complete media supplemented with oleate and ergosterol.

Although mutants lacking the capacity to synthesize sterol and requiring only sterol for growth in aerobic cultures have as yet not been isolated, ergosterol is not synthesized under anaerobic conditions (93) and would be expected to be required for growth under conditions rigorously excluding oxygen. Andreason and Stier (4) demonstrated that under strictly anaerobic conditions yeast exhibited a vitamin like requirement for ergosterol. They found that in the absence of exogenous ergosterol growth in a defined medium in the absence of oxygen gave but a one to two-fold increase in cells. With the addition of ergosterol a cell yield one-third that reached in aerated cultures was obtained. However, this requirement for sterol was not limited to ergosterol. Cholesterol was found to promote anaerobic growth of the yeast, although to a lesser degree. In a subsequent communication (5) the authors demonstrated that in addition to ergosterol, oleic acid was required for growth under strictly anaerobic conditions. They noted that the emulsifier Tween-80 was capable of supplying the required oleic acid for anaerobic growth, whereas Tween-20, Tween-40 or Triton could not. Additionally

they showed that both oleate and ergosterol depress the cell yields obtained in aerated cultures, when these lipids are added at concentrations which are optimal for anaerobic growth.

The addition of various sterols has been reported to have varying effects on the growth of microorganisms. Cholesterol, ergosterol and vitamin D_2 have been reported to have an inhibitory effect on bacterial growth (66) while ergosterol, stigmasterol, progesterone, squalene, dihydrocholesterol acetate and dihydrocholesterol palmitate inhibited the growth of the enteric amoebae Entamoeba histolytica (9). It has been observed that cholesterol inhibited and ergosterol stimulated the growth of the pathogenic fungus Trichophyton rubrum (10). Tauson (92) found that ergosterol added to cultures of Saccaromyces carlsbergensis increased the cell yield by 6% of the dry weight, fermenting activity by 16% to 19% but that the rate of dehydrogenating glucose and succinic acid was decreased. Matkovics and Sipos (54) found that the addition of ergosterol or cholestrol to culture media increased the growth of fungi. They reported that the addition of the sterol changed the surface tension of the medium, thereby causing better aeration for growth. Matkovics and Konschansky (53) reported that ergosterol caused a slight increase in the growth rate of Penicillium chrysogenum, but had no effect on the total dry weight of the mycelium. They demonstrated that the ergosterol had only a slight effect on the surface tension of

the medium but markedly inhibited dehydrogenase formation. Festenstein (24), working with rat liver slices, demonstrated that the addition of ergosterol or ergocalciferol appreciably inhibited anaerobic glycolysis.

Ergosterol has also been implicated for a role in certain enzymatic processes. Oparin and co-workers (62), using purified trypsin, demonstrated that the addition of crystalline ergosterol to trypsin solutions resulted in the formation of a dimeric trypsin-ergosterol complex which was stable for 48 hours and which was significantly more reactive on egg albumin and horse serum albumin than was the monomeric enzyme. They suggest that the ergosterol might play some kind of a regulatory role in intact yeast. Talalay and Williams-Ashman (89) have found that estradiol is capable of activating a transhydrogenase from human placenta catalyzing the reduction of isocitric acid to α-keto glutaric acid. The sterol is postulated to participate in a reversible reaction involving the reduction of estrone to estradiol by TPNH, the estradiol being then reoxidized by DPN^+. The steroids are seen here to be acting as coenzymes in the oxidation and reduction of pyridine nucleotides. Although oxidation of the 3-β-hydroxyl group of ergosterol by DPN^+ might suggest such a role for ergosterol in yeast, no keto steroids have been reported to exist in yeast nor is it known whether or not yeast possess a transhydrogenase system. Deborin and co-workers

(19) have shown that phosphoglyceraldehyde dehydrogenase can bind minute quantities of ergosterol. This binding is found to lead to a change in viscosity, optical rotation and the molecular weight of the enzyme. The authors postulate that ergosterol may play a role in regulating the activity of the enzyme, affecting hydrogen transfer to DPN^+.

Probably the most completely defined function of sterols in microorganisms has been that of the role sterols have been demonstrated to play in pleuropneumonia-like organisms. Smith and Lynn (80) observed that certain strains of pleuropneumonia-like organisms required the addition of exogenous sterols to the medium for growth. Using cholesterol as a reference standard, they showed that β-sitosterol, ergosterol and dihydrocholesterol added to the growth medium were capable of supporting growth of the organisms. They found that sterols possessing only subtle changes from cholesterol in molecular configuration supported growth and generalized that the 3-hydroxyl group, the cylcopentanphenanthrene ring, and the side chain of cholesterol are absolute requirements for growth. Even minor modifications caused a reduction in growth promoting activity. Subsequently Smith (76) demonstrated that an esterase distinct from lipase activity and capable of synthesizing cholesterol esters of fatty acids and of hydrolytic and thiolytic cleavage existed in all strains of the organism. This esterase and the large

majority of the nonsaponifiable lipid of the cells was located in the cell membrane fraction. Smith and Rothblat (81) observed that cholesterol-4-^{14}C was removed from supernatant media by both growing and resting cells and that this sterol was found to be incorporated solely into the nonsaponifiable lipid fraction. The uptake of sterol appeared to be an irreversible process being removed only by solvent extraction. The adsorption process was affected by time, temperature, cell concentration and pH, but not by respiratory poisons and the destruction of protein end groups on the cell surface. It was also observed that cholesterol taken up by saprophytic, nonsterol requiring strains appeared to be converted into some nonsaponifiable lipid which was unreactive with digitonin. Treatment of parasitic sterol requiring strains with digitonin caused lysis of the cells. The fate of adsorbed sterols was further investigated by Rothblat and Smith (70). It was found that all of the cholesterol removed from the medium by a nonfermentive sterol requiring strain remained unchanged except for a portion of it being esterified. On the other hand, a sterol requiring fermentive strain converted a portion of the cholesterol to cholesteryl-β-D-glucoside, the amount of glucoside formed being governed by the amount of glucose supplied in the growth medium. This cholesteryl glucoside was found to be formed by a fermentive nonsterol requiring strain only when grown in the presence of exogenous cholesterol. Analysis of the

nonsaponifiable fraction of nonsterol requiring strains in the absence of exogenous sterol revealed the presence of three carotenoid pigments. The most nonpolar of these was identified as the hydrocarbon neurosporene, the second appeared to be a hydroxylated neurosporene, and the most polar pigment a carotenol-glucose complex. Subsequently, it was demonstrated that while saprophytic nonsterol requiring strains could incorporate acetate-1-^{14}C and mevalonic acid-2-^{14}C into their nonsaponifiable fractions, parasitic sterol requiring strains could not. None of the nonsaponifiable lipid synthesized by the saprophytic strains appeared to be 3-β-hydroxy sterols. Smith (77) has shown that ergosterol and cholestanol can substitute for cholesterol. Smith (79) has further demonstrated that the carotenol occurring in these strains existed as the free alcohol, esterified with fatty acid, and in glycosidic linkage with glucose. It was noted that the only volatile fatty acid esterified to the carotenol in fermentive organisms was acetate whereas in nonfermentive organisms the carotenol was also esterified to butyric acid. In a review on the role of sterols in the growth and physiology of pleuropneumonia-like organisms, Smith (78) postulates that in nonsterol requiring strains carotenols carry out a bifunctional role; the maintenance of structural integrity of the cell membrane and as intermediates or carriers of substrates and metabolic endproducts for transport across the cell membrane.

The carotenol acts to carry glucose from the medium across the membrane and to carry the metabolic product, acetate, back out. Strains which have lost the capacity to synthesize carotenols can incorporate sterols of the proper molecular configuration to perform a similar function.

At the present time, there has been no report of the isolation from yeast of an ergosteryl glucoside. However, experiments dealing with the protective action of sterols against inhibition by the polyene antifungal antibiotic, nystatin, indicate a major function for sterols in the yeast cell membrane. Lampen and co-workers (42) found that nystatin is bound by yeast protoplasts and that 90% of the bound nystatin and of the sterol is located in the cell membrane fractions. Digitonin treatment was found to remove 80 to 85% of the bound nystatin and to cause lysis of the protoplasts. Osmotic lysis did not release the antibiotic. It was found that isolated cell membranes rapidly bound nystatin and a rough correlation was noted between sterol content and nystatin binding capacity. The authors conclude that in all probability, membrane sterols constitute the critical nystatin binding sites. That sterols perform a function related to permeability in the yeast membrane is also suggested by the use of nystatin treatment of whole cells to obtain "permeabilized" yeast which are hyperpermeable to various substrates included in the medium (8).

Wild type strains of Saccharomyces cerevisiae possess both fermentive and respiratory mechanisms as a means of deriving energy. Under anaerobic conditions a fermentive metabolism is carried out while in the presence of oxygen a respiratory cycle is induced. Loss of respiratory competency does not result in a loss in viability but rather in respiratory deficient organisms as a result of either a nuclear mutation or through the loss of a cytoplasmic determinant. These mutants were first recognized by their slow growth and small colony size on glucose and were given the name petites (60). Petite mutants resulting from the loss of a cytoplasmic determinant exhibit a complete lack of cytochromes A, B and oxidase and of succinic dehydrogenase. They have reduced levels of cytochrome E, NADH linked cytochrome C reductase and α-glycerophosphate dehydrogenase. They are found to have increased levels of alcohol dehydrogenase and often soluble cytochrome C and are found to possess cytochrome A_1 and malic cytochrome C reductase, which are not found in normal yeast (22, p. 24). Petites are recognizable by their colony size, their inability to grow on nonfermentable substrates (22, p. 21) and by overlay with triphenyltetrazolium chloride (59). The cytoplasmic petite mutation can be induced by a variety of mutagenic agents. The most effective of these is acriflavine, which is an acridine antiseptic dye which contains euflavine, 3, 6 Diamino-N-methyl acridine chloride. Other chemical agents include

triphenyltetrazolium chloride, sodium cyanide, sodium azide, dinitrophenol, and cupric, nickel, cobalt and manganese ions. Irradiation with ultraviolet light and growth at high temperatures will also induce the mutation (60).

When yeast cells are shifted from an anaerobic to an aerobic environment, the enzymes necessary for the respiratory apparatus must be induced, and it has been shown that molecular oxygen is the inducing agent and that the magnitude of this inductive action is increased the closer a particular enzyme along the respiratory chain is to oxygen (60).

The process of induction of respiratory enzymes by oxygen can be inhibited by acriflavine at the same concentration that induces the petite mutation. It inhibits the synthesis of the enzymes concerned and has no effect on the activity of the enzymes already present. If the system has already been induced, respiration will proceed in the presence of acriflavine as long as cellular division doesn't occur (60). It has also been observed that the respiratory mutation cannot be induced in a nonproliferating cell suspension (22, p. 27). That the acriflavine inhibits the synthesis of the enzymes concerned with respiration is supported by the observation (44) that DNA in solution with acridine, euflavine or acridine orange leads to increased viscosity and sedimentation values of the DNA. X-ray diffraction patterns of these complexes suggest a considerable modification of the

usual helical structure, and it is inferred from this that the polyaromatic dye molecules are intercalated between adjacent nucleotide bases, resulting in an extension and partial unwinding of the DNA. Such an effect might considerably inhibit both the replication of the DNA and messenger transcription.

The existence of a relationship between ergosterol production and aerobiosis has been previously noted (50, 64). Klein (35) proposed that lipogenisis may be an adaptive response to aeration and that there exists a direct relationship between oxidative metabolism and sterol synthesis. Parks and Starr (64) observed that in cultures grown under microaerobic conditions and subsequently aerated in media lacking a nitrogen source, an initial increase in the sterol content of the cells was followed by a depression and subsequent increase when the time of incubation and sterol content were followed. This depression in sterol content was found to coincide with the exhaustion of glucose from the aeration medium and the initial period of utilization of accumulated ethanol. Furthermore, it was found that the higher the glucose concentration employed in the aeration medium, the later this depression in sterol content would occur in the course of analysis. Enzyme inhibitors such as acriflavine, cyanide, dintrophenol, sodium azide, and cupric, cadmium and maganese ions, known to stimulate the induction of petite mutations, were assayed for their effect on sterol synthesis in similarly

aerated suspensions. With the exception of sodium azide, a marked reduction in the sterol content of batch cultures aerated in the presence of these inhibitors was observed. Respiratory petite mutants, isolated from cultures grown at high temperatures, were found to have retained the capacity to synthesize ergosterol. Considerable variation in the amount of sterol formed was noted, with some clones yielding more sterol than the wild type parent strains. Kovac and co-workers (41) found that when anaerobically grown wild type strains were aerated in the presence of acriflavine or chloramphenicol, the cells did not become respiratory competent. It was determined that when this occurred the content of phospholipids and of total fatty acids increased as in adapting control cells whereas there was a reduction in sterol synthesis. Petite strains showed the same pattern of lipid composition as did the wild type parent except that the phospholipid content was lower in anaerobically grown petites than in the anaerobically grown wild type cells. The authors comment that the observed lower lipid content of the anaerobically grown cells as compared to aerobically grown cells cannot be due to a limited supply of energy for lipogenesis under anaerobic conditions since the content of lipids in petite cells was subject to the same variations.

It has been demonstrated by a number of investigators that the carbon source and lipid composition of the medium has a marked

effect on the synthesis of respiratory enzymes and upon the formation of the cellular structures associated with aerobic metabolism. Polakis and co-workers (65) demonstrated that yeast grown on different concentrations of glucose went through an adaptation to aerobiosis wherein the cells at 12 hours of growth were found to be able to oxidize glucose and ethanol. The cells, however, were unable to oxidize acetate at this stage and electron microscopy revealed an absence of mitochondria and the presence of intracellular vacuolor structures. After 24 hours of growth the cells were able to oxidize acetate and had maximal rates of glucose and ethanol oxidation, the activity of the tricarboxylic acid cycle and respiratory enzymes being maximal. At this stage large numbers of mitochondria were present and the vacuoles were largely absent. This oxidative activity was lost at 36 hours of growth. In conjunction with this increase and subsequent decrease of respiratory activity of yeast during different stages of growth, it has been demonstrated that yeast grown microaerobically by the method of Klein (35) exhibit a maximal capacity to produce both ergosterol and ergosterol esters when aerated in nonproliferating cultures after 48 hours of growth, when the cells were in the stationary phase. This ability was rapidly lost after 48 hours of growth (49). Tustanoff and Bartley (98) have found that the ability of anerobically grown cells to adapt to aerobiosis is critically dependent upon the concentration

and time of exposure to glucose during growth. They found that the lower the concentration of glucose used in growing the yeast, the earlier subsequent respiratory capacity was attained and that this respiratory activity was greater the lower the concentration of glucose used. It was demonstrated that the consumption of glucose from the medium was linear and that this was reflected by a linear production of ethanol. Respiration was found to commence when the glucose in the medium had fallen to less than one-half of the original concentration and was at a maximum only when the glucose had been completely exhausted. Growth on the slower fermented sugar galactose was found to allow the cells to respire almost immediately upon subsequent aeration and the inhibitory effect of glucose was partially relieved by inclusion of the medium of casein hydrolysate. The authors suggest that the synthesis of respiratory enzymes in yeast is subject to a catabolite repression. Morpurgo and co-workers (56) observed that the inclusion of ergosterol in statically grown cultures resulted in cells possessing typical mitochondrial structures which were, however, less well developed than mitochondria from aerobically grown cells. Cells grown without exogenous ergosterol, on the other hand, had mitochondrial vessicles completely lacking in internal structures.

An important point to be considered when studying the induction of respiratory enzymes in cultures grown under anaerobic

conditions has been brought up by Somlo and Fukuhara (83). Using nitrogen of the highest commercial purity, it was demonstrated that sufficient oxygen is present to partially induce the aerobic system of yeast in cultures flushed with such nitrogen. Using additional oxygen removing methods, it was shown that as the oxygen content of the nitrogen is further decreased, the increased respiratory activity observed by Tustanoff and Bartley (98) of cells grown on galactose as compared to those grown on glucose is diminished until the spectral bands of the cytochromes and the activity of the respiratory enzymes of the two cultures are equal. Thus one must consider the bulk of the literature purporting to deal with anaerobic cultures to be actually microaerobic cultures. It should be noted here that the catabolite repression of respiratory enzymes observed by Tustanoff and Bartley is no less valid, but rather cannot be considered a phenomenon of strictly anaerobic systems.

Lukins and co-workers (47) have observed that yeast grown anaerobiocally on galactose without lipid supplements contain only trace amounts of succinic dehydrogenase and few mitochondrial vessicles. The inclusion in the medium of Tween-80 and ergosterol produced a profound change in succinic dehydrogenase activity and the cytological characteristics of the cells. Enzymic activities were up to 15% of that of aerobically grown cultures. Cells grown anaerobically on glucose without lipid supplements also did not

contain detectable amounts of enzymes or differentiated membranes. However, in the case of the glucose grown cells, inclusion of Tween-80 and ergosterol in the medium did not result in detectable amounts of succinic dehydrogenase, although some very slight cytological differentiation was noted. The authors conclude that the formation of succinic dehydrogenase and associated mitochondrial membranes is dependent upon the lipid composition of the medium and is subject to a catabolite repression.

As noted previously (60), respiratory deficient petite strains can be obtained by growth of yeast cultures at elevated temperatures. Ycas (103) and Sherman (73) first observed that growth at 40°C resulted in increased mutation to cytoplasmic petites. Sherman (74) observed that at 40°C yeast cultures entered a death phase, similar to thymine-less death, which could be averted by the addition of either yeast extract or oleic acid. Loginova and co-workers (45) showed that ergosterol, Tween-80, and oleic acid increased growth and CO_2 production of yeast grown at 39°C to 40°C. Starr and Parks (84) measured sterol production of microaerobically grown cells in aerated nonproliferating suspensions and observed that sterol synthesis was maximal at 30°C, the optimal growth temperature, and diminished rapidly as the temperature of incubation was increased. At temperatures of 40°C and above synthesis is initiated but declines with prolonged incubation. Supplementation

of the medium with oleic acid and ergosterol permitted cell growth and prevented the death phase described by Sherman. Addition of Tween-80 without ergosterol gave an initial burst of growth followed by cell death and a decline in the optical density of the culture, when grown at 40°C. It was observed that at 45°C to 50°C the cells in the absence of lipid supplements were almost completely lysed. Parks and Starr (64) have demonstrated that ergosterol, provided either in an albumin complex or in Tween-80, reduced the number of petite colonies obtained from growth at 40°C by one-third to one-half the numbers obtained in the control without lipid supplements.

In considering the cause of petite induction in yeast, Sarachek and Fowler (72) postulate that although the lipid composition of wild type and petite strains is similar, the fact does not exclude the possibility that a "temporary disturbance" in lipogenisis might be involved in mutant formation. Working on this hypothesis, the percentage of petites occurring in a wild type population of yeast exhibiting a partial requirement for pantothenate was assayed. They found that when this strain is grown aerobically in the absence of exogenous pantothenate, during the transition period from the logarithmic phase of growth into the stationary phase, the cells are converted <u>en masse</u> to respiration deficient mutants. It was found that this conversion does not occur in the presence or absence of

of pantothenate in anaerobic cultures. The individual inclusion of acetate, ergosterol and linoleic, palmitic, stearic and oleic acids in the growth medium revealed that oleic acid and acetate effectively prevented the conversion. It was concluded that the demand for coenzyme A placed on the cell by the development of the respiratory apparatus could not be met by the deficient cells and, as a direct result of this, the mutation was induced.

Current theory based on substantial supporting genetic data predicts that respiratory incompetency results from the "functional" loss of an autonomously reproducing cytoplasmic factor. Parks and Starr (64) suggest that ergosterol could quantitatively control mitochondria production since under conditions limiting its availability fewer functional particles would be made. They note that petite induction occurs only in growing cells and that under artificial conditions which severely limit ergosterol production, but yet permit cell proliferation, functional respiratory particles or particle precursors would be produced at such a severely reduced rate that daughter cells would not receive the cytoplasmic unit needed for insuring respiratory sufficiency. Cells not requiring these functional particles would not be aerobically functional, yet would have the capacity for sterol synthesis under normal conditions. As observed by Ephrussi and Hottinguer (23), "it must be admitted that a distinction cannot be made between a true loss and an irreversible

mutation affecting the heterocatalytic function of the particles in petite induction." The demonstration of Parks and Starr that ergosterol synthesis is inhibited at high growth temperatures, the fact that petites are induced by such growth, and that exogenous ergosterol relieves this induction to a significant degree speak for the loss of some cytoplasmic component in the process of petite induction.

If such a cytoplasmic determinant in fact exists, one might suspect that it would be controlled by DNA. Tewari and co-workers (94) isolated from yeast mitochondria a DNA differing significantly from nuclear or other cytoplasmic DNA. It was suggested that this component may play a role in the cytoplasmic control of respiration in yeast. Subsequently, Moustacchi and Williamson (58) compared the mitochondrial DNA of both normal and cytoplasmic petite strains of yeast. They observed in wild type strains a DNA fraction corresponding to that of Tewari and co-workers, termed the β fraction, and another, heavier, fraction which they termed the γ fraction. It has been shown in wild type strains that in the first eight hours of growth the β fraction fell to approximately 3% of the total DNA but reached a maximum of 20% as the cells enter the stationary phase. The γ fraction was found to vary in a similar manner, the fraction varying from 1 to 11% of the total DNA. In cytoplasmic petite strains however, the β fraction could not be detected although

the γ fraction was still present. It was concluded that the β fraction is responsible for cytoplasmic determinancy of respiratory competency. No role was postulated for the γ fraction. It was also suggested that the DNA fractions from the mitochondria are under independent control from the nuclear DNA and that the glucose induced repression of the relative respiratory rate in the early stages of growth on glucose may be a consequence of direct repression of the synthesis of mitochondrial DNA rather than repression of enzyme synthesis.

In a subsequent communication, however, Mounolou and coworkers (57) contend that the cytoplasmic petite mutation is not due to the loss of a mitochondrial DNA fraction but rather to a change in its buoyant density. Although the fraction of Moustacchi and Williamson was not found in cytoplasmic petite strains, another, lighter, DNA fraction was detected which is not present in wild type strains.

That the actual site of the control and synthesis of respiratory enzymes is in the mitochondrion has been demonstrated by Clark-Walker and Linnane (12). They found that high concentrations of chloramphenicol in the growth medium completely inhibited the formation of cytochromes A, A_3, B and C_1, partially inhibited succinic dehydrogenase formation, but had no effect on soluble cytochrome C synthesis. Additionally, a marked reduction of

mitochondrial cristiae formation was noted in the presence of the drug. In glucose repressed yeast, chloramphenicol had little effect on outer mitochondrial membrane formation or on the synthesis of malate dehydrogenase or fumarase. All of these are markedly decreased on growth of glucose derepressed yeast in the presence of the drug. It was also found that the drug had no effect on petite strains. The authors conclude that since chloramphenicol interacts only with the 70S ribosomal system characteristic of bacterial and mitochondrial synthesizing systems, those components whose synthesis is inhibited by the drug must be synthesized by the mitochondrial protein synthesizing system.

Another drug found to inhibit both the synthesis of the respiratory apparatus and sterol formation in yeast is the convulsant drug pentamethylenetetrazol, or Metrazol. Alexander and co-workers (3) found that the inclusion of Metrazol in the growth medium caused aerated yeast to behave as if grown under microaerobic conditions. It was demonstrated that yeast grown in the presence of the drug possessed fewer mitochondria which were cytologically different from those isolated from control cells and similar to those from yeast grown anaerobically under nitrogen. The level of sterol was reduced to that of yeast grown under microaerobic conditions and this reduction was inversely proportional to the Metrazol concentration. Removal of the drug gave immediate synthesis of sterol similar to that observed in

the aeration of anaerobically grown cells.

MATERIALS AND METHODS

Cultures

Strain 22 B, a haploid methionine requiring clone, was used in the majority of these experiments. For mutation studies strain 3701 B, a haploid uracil requiring strain was employed. Stock cultures were maintained on YCM agar medium at 5°C and transferred once each month.

Media

The composition of growth media used in these experiments is shown in Table 1. When YMAF medium was used to obtain microaerobic cultures, 1700 ml of the medium (minus carbon source) was placed in a two liter flask and autoclaved. Immediately after autoclaving, the glucose (autoclaved separately) was added and the flasks allowed to cool before inoculation. The defined medium of Wickerham (100) was employed when required. A medium, designated WCY, was used in mutation studies and consisted of Wickerham's complete medium plus 10% v/v YCM medium. The aeration medium used for experiments with nonproliferating cell suspensions consisted of 1% glucose and 0.1 M KH_2PO4.

Materials

Ergosterol and cholesterol were purchased from Sigma Chemical Company and recrystallized from ethanol before use. Primary labeled ^{14}C hexadecane was a gift from Dr. Len Gawel, ^{14}C labeled pulegone a gift from Dr. W. D. Loomis, and ^{14}C labeled squalene a gift from Dr. D. Baisted. Pentamethylenetetrazol (Metrazol) was donated by the Knoll Corporation and chloramphenicol by the Parke-Davis Company. The following materials were commercial products: Blue dextran, Sephadex G-50, and glass chromatography columns from Pharmacia Fine Chemicals, Inc.; Silica Gel G from Brinkmann Instruments, Inc.; digitonin from Calbiochem; reagent grade dimethylsulfoxide (DMSO) from J. T. Baker Chemical Company; glycogen isolated from oysters from Sigma Chemical Company; trehalose dihydrate from Calbiochem. Nitrocellulose membrane filters were type B 6, obtained from Schleicher and Schuell Company. All other materials were obtained from commercial sources at the highest available purities.

Growth conditions for cells

Microaerobic cultures employing YMAF medium were obtained by innoculating the YMAF cultures from a 16 hour statically grown TCA broth culture and incubating statically at 30°C for 48 hours.

Resting cell suspensions were prepared for aeration by allowing the microaerobically grown cultures to settle in the cold, centrifuging, washing four times with ice cold phosphate buffer (6.53 g of K_2HPO_4 and 8.51 g of KH_2PO_4 per liter, final pH 6.6) and resuspending the cells in aeration medium. For comparison of glucose and galactose grown cells the carbon source in the YMAF medium was employed at a concentration of 5%. Equal inocula were made into the two media on the basis of turbidity of the washed and resuspended 16 hour TCA broth cultures employing the respective carbon source at a concentration of 2%.

Extraction and analysis of sterols

Routine saponification of cells was performed by adding an equal volume of 40% KOH and a boileezer (Fisher Scientific Company) to a yeast suspension in a glass stoppered extraction tube (Kontes Glass Company). The tubes were covered loosely with aluminum foil and placed in an Arnold sterilizer for six hours after which they were cooled and extracted with three 10 ml volumes of light (30^{o} - 60^{o}) petroleum ether. The extracts were evaporated to dryness under a stream of nitrogen.

The quantity of sterol present was determined by measuring the methyl group from methyl-^{14}C-methionine (New England Nuclear Company) incorporated into ergosterol. This has been shown to

occur as the 28th carbon on the side chain of the sterol through transmethylation by S-adenosylmethionine (63). That the methylated nonsaponifiable products obtained from this procedure are sterols has been demonstrated previously (97). In order to further verify that the methylated compounds in the nonsaponifiable fraction and in the acid hydrolyzed fractions are indeed sterols, the crude extracts from saponified and acid hydrolysed cells previously aerated in the presence of methyl-^{14}C-methionine were analyzed by thin layer chromatography and digitonin precipitation in the following manner.

A resting cells suspension was prepared as described above and 10μc of methyl-^{14}C-methionine were added. The cells were aerated on a rotary shaker for four hours after which the cells were centrifuged, washed three times with cold distilled water, and resuspended in 95 ml of distilled water. The suspension was then distributed in 30 ml volumes and treated as follows. To sample A, 30 ml of 40% KOH were added and the cells saponified in the routine manner for six hours, cooled and then extracted with four 50 ml volumes of light petroleum ether. To sample B, 30 ml of 0.1 N HCL were added and the suspension incubated at 30°C for one hour. The suspension was then centrifuged, and supernatant decanted and discarded, the volume brought to 30 ml with distilled water, and 30 ml of 40% KOH were added. The sample was then saponified

in the routine manner, cooled, and extracted. To sample C, 30 ml of 0.1 N HCL were added and the suspension placed in an Arnold sterilizer for one hour. The suspension was then cooled, the pH adjusted to 10 with KOH, and the sample extracted. The extracts from the samples were evaporated to dryness under nitrogen. To samples A and B, 20 ml volumes of light petroleum ether were added; to sample C, 10 ml of petroleum ether were added. These samples are henceforth referred to as samples A, B, and C, respectively.

For digitonin precipitation and specific activity determination 0.25 ml volumes of each sample were removed to scintillation vials for counting, to 30 ml Corex centrifuge tubes containing 1.0 mg carrier ergosterol for digitonin precipitation, and to colorimeter tubes for Liebermann-Burchard color determination. All procedures were performed in duplicate.

A) Scintillation counting: The control samples were evaporated to dryness under N_2 and assayed for radioactivity by adding 20.0 ml of scintillation fluid (3.0 g of 2,5 diphenyloxazole (PPO) plus 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) per liter of toluene) to each sample in a glass scintillation vial. The samples were counted in a Packard model 3000 Tri-Carb Liquid Scintillation Spectrometer. A ^{14}C-toluene standard was employed to monitor the counting efficiency of the instrument on all assays.

B) Digitonin precipitation and recovery of sterol: The extracts and carrier sterol were dried under N_2 and redissolved in 10 ml of acetone:absolute ethanol (1:1). To this 10 ml of 0.5% digitonin in 50% ethanol were added, the tubes mixed, and stored overnight at -20°C. The tubes were then centrifuged at 17, 300 x g in a Sorvall Model RC-2 refrigerated centrifuge. The supernatant liquid was decanted and discarded and the digitonides washed with two 5 ml volumes of acetone:diethyl ether (1:1) and one 5 ml volume of diethyl ether. The digitonides were then dried under a stream of N_2. Sterol was cleaved from the digitonides with dimethylsulfoxide (33). Five ml of DMSO were added to each tube, the tubes covered with foil and heated in an Arnold sterilizer for 30 minutes. The sterols were then extracted with four 10 ml volumes of petroleum ether and the radioactivity assayed as described above. A control determination run concurrently in sextuplicate on 1.0 mg quantities of standard ergosterol and assayed colorimetrically by the Liebermann-Burchard procedure showed the percent recovery of sterol by this method to range from 92 to 98% with a mean recovery of 96% of the initial sterol.

C) Liebermann-Burchard colorimetric determination of sterol: Dried sterol extracts were dissolved in 3.0 ml of chloroform and 2.0 ml of a 19:1 solution of concentrated sulfuric acid:acetic anhydride added and the samples mixed. Color development was

allowed to proceed for 15 minutes after which the optical density at 625 mμ was determined against ergosterol standards.

The results of these determinations are given in Table 2.

For radioassay and thin layer chromatography Silica Gel G mixed as 50 g Silica Gel G to 110 ml distilled water was spread on plates to a thickness of 0.375 mm with a Desaga-Brinkmann spreader and activated for 30 minutes at 100°C prior to use. The plates were developed in a redistilled benzene:ethyl acetate (5:1) solvent system in the dark. Sterol was located on the plates either by spraying with 20% antimony pentachloride in chloroform (7), or with Liebermann-Burchard reagent. In order to correlate sterol color with radioactivity, 75 μl volumes of samples A and B and 125 μl of sample C were spotted on separate 50 x 200 mm plates. Following development the plates were monitored with a Vanguard Model 885 thin layer plate scanner. The plates were then sprayed with sterol localization reagent. Figure 2 shows the resulting radiochromatograms. The radiochromatogram for sample A is not shown, since it is identical to that of sample B. It can be seen that the only radioactivity in the saponified samples (A and B) corresponds to the color spot revealed with antimony pentachloride and traveling with an Rf of 0.40, the same Rf observed with standard ergosterol in this system. This spot also gave positive sterol color with Liebermann-Burchard reagent. Two spots giving positive sterol color with antimony pentachloride

Table 1. Composition of Growth Media

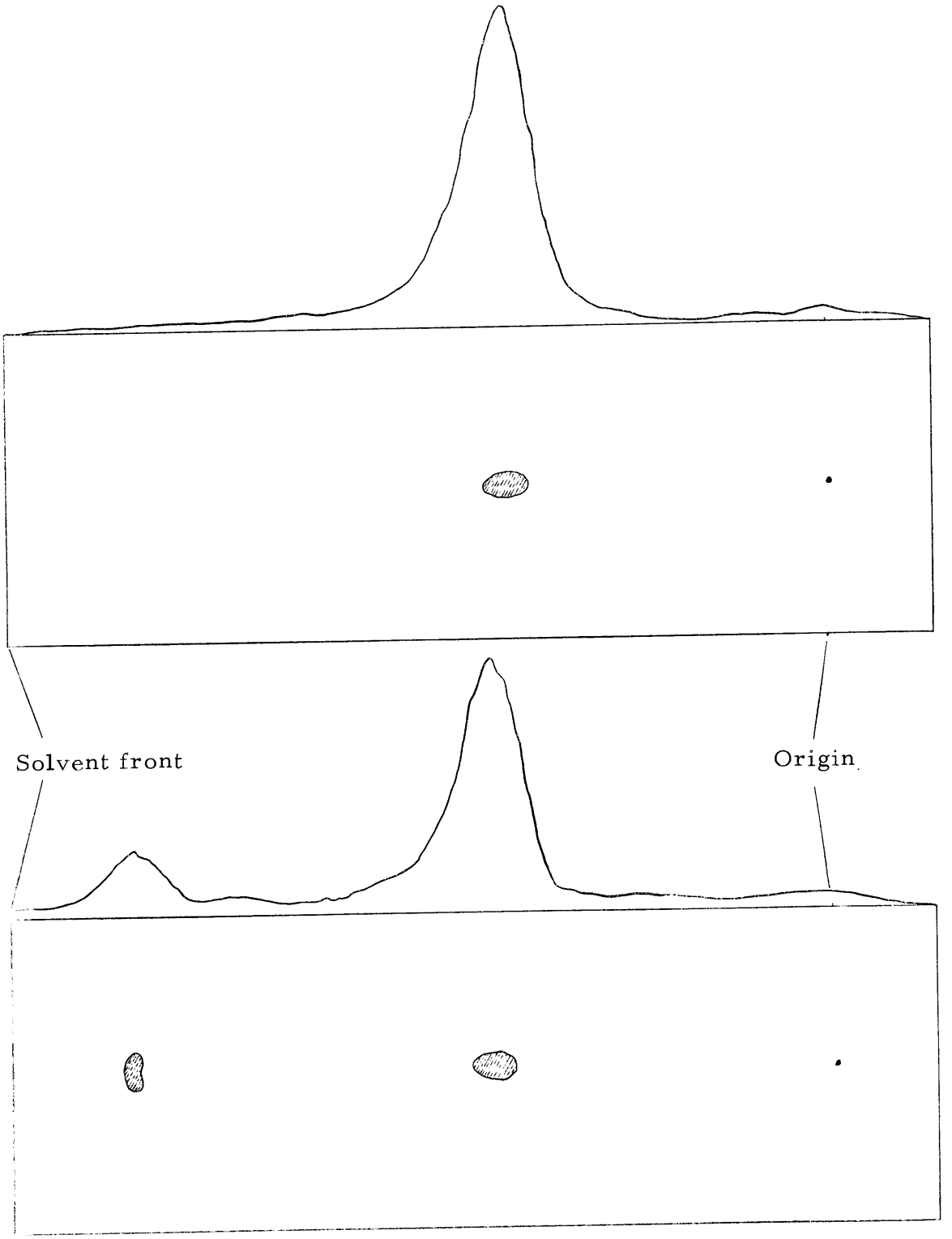
Component	TCA	YCM	YCC	YMAF
	%	%	%	%
Yeast extract	0.5	1.0	2.0	0.2
Tryptone	1.0	2.0	1.0	---
Glucose	2.0	2.0	2.0	2.0
NH_4Cl	---	---	---	0.1
KH_2PO_4	---	---	---	0.92
K_2HPO_4	---	---	---	0.55

Glucose or galactose was utilized as indicated in the text. When agar was used a 2% concentration was employed.

Table 2. Percent Radioactivity Recovered Following Digitonin Precipitation and Specific Activity of Extracted Sterol

Sample	A	B	C
DPM before precipitation	63,800	78,200	66,300
DPM recovered as sterol	59,600	73,500	50,900
Percent DPM recovered	93.4	94.0	76.8
μg sterol	94	110	92
Specific activity: DPM/μg sterol	679	711	720

Sample A refers to a petroleum ether extract of cells subjected to routine saponification. Sample B refers to a petroleum ether extract of cells treated with cold 0.1N HCl followed by routine saponification. Sample C refers to a petroleum ether extract of cells treated with hot 0.1 N HCl, the pH being adjusted to 10 before extraction. Duplicate 0.25 ml volumes of each sample were removed for liquid scintillation counting, for digitonin precipitation, and for Liebermann-Burchard color determination. DPM = disintegrations per minute.


Solvent front
Origin

and Liebermann-Burchard reagent were observed in samples treated as in procedure C. Free ergosterol and an additional component traveling with an Rf of 0.86 corresponding to an ester of ergosterol are present. That this faster migrating substance corresponds to an ester of ergosterol is readily demonstrated by saponifying sample C. Rechromatography in this same system following saponification reveals only free sterol. In order to further validate the existence of ester, authentic ergosteryl palmitate was synthesized using the techniques of Swell and Treadwell (88) utilizing the acyl halide derivative of palmitic acid. Nuclear magnetic resonance and infrared spectroscopy of the synthesized product by Dr. F. T. Bond of the O. S. U. Department of Chemistry, and a determined melting point of 107.5°C (103) were used to establish the identity and purity of the synthetic ester. This ester was found to have an identical chromatographic migration as the suspected ester component in sample C.

The occurrence of ester in sample C explains the lower recovery of radioactivity following digitonin precipitation with respect to that observed with samples A and B; that is, 76.8% recovery for sample C and 93.4% and 94.0% recovery for samples A and B, respectively. Since the ester would not be precipitated by digitonin, the percentage of labeled sterol occurring as ester in sample C can be determined in correcting the 76.8% recovery of activity value for

the 94% recovery of free sterol observed with samples A and B. This correction gives the percentage ester in sample C as 18%. In order to substantiate this determination, the percent ester was determined by integrating the areas under the separated sterol peaks from the radiochromatogram of sample C. The percent ester determined in this manner was 16%. The 2% difference is within experimental error.

Preparation of the sterol complexing agent from yeast extracts

Ninety g of Fleischmann's Dry Yeast were aerated in aeration medium for four hours, centrifuged, washed in distilled water, resuspended in distilled water (total volume, 400 ml), and refluxed for four hours. The suspension was filtered while still hot through Whatman No. 2 filter paper and through a nitrocellulose filter to give a clear yellow solution which was lyophillized and resuspended in distilled water to give a 15% w/v solution. Twelve ml of this solution was chromatographed on a Pharmacia glass column, 2.5 cm I. D., packed to a height of 31 cm with Sephadex G-50. The column had a void volume of 55 ml as determined with blue dextran. The column was eluted with 0.1 M phosphate buffer (pH 6.6) at a flow rate of 3.0 ml/min. After collection of the first 40 ml of eluent, samples were collected at one minute intervals for 15 minutes. Each sample was combined with an equal volume of 95% ethanol,

stored at -20°C overnight and the precipitate sedimented by centrifugation. This high molecular weight fraction was dissolved in distilled water and reprecipitated with an equal volume of 95% ethanol. The precipitate was then dried by lyophillization.

Preparation of ergosterol complex

To a solution of 15% w/v Difco yeast extract, enough of a 1.0 mg/ml solution of ergosterol in absolute ethanol was added to give a final concentration of 150 μg/ml. Uncombined sterol was removed by hexane extraction, and 50 ml of solution were passed through a column of Sephadex G-50 (4 cm I. D., height of 51 cm). Elution was performed with 0.1M phosphate buffer. The opalescent solution emerging just after the void volume of the column was examined and found to contain ergosterol.

Isolation of sterol

Methanolic pyrogallol saponification was carried out by adding 0.5% pyrogallol in absolute methanol-60% KOH-absolute methanol 3:2:3 to precipitated or lyophillized column fractions in extraction tubes fitted with condensers and refluxing for 1.5 hours. Methanol pyrogallol acid hydrolysis was performed in the same manner except for the substitution of an equal volume of 1N HCl for the 60% KOH. Simple acid hydrolysis consisted of making samples 0.1N with

respect to HCl by the addition of 0.2N HCl and heating for one hour in an Arnold sterilizer, adjusting the pH to 10 with KOH and extracting the lipid with n-hexane.

Sterol was recovered from the Sephadex column fractions by precipitation with digitonin or cleavage with DMSO. Precipitation with digitonin consisted of combining the column fractions containing sterol with different ratios of 0.5% digitonin in 50% ethanol. The digitonides were stored at -20°C overnight, centrifuged, washed twice with acetone:diethyl ether 1:1 and once with diethyl ether, and dried under nitrogen. The digitonides were dissolved in 3.0 ml volumes of glacial acetic acid, 2.0 ml of Liebermann-Burchard reagent added, and the optical density at 625 mμ measured against standard ergosterol digitonide.

When cleavage with DMSO was used, the column fractions containing sterol were first combined with equal volumes of 95% ethanol. The samples were well mixed and stored overnight at -20°C. The tubes were then centrifuged at 14,000 x g for 15 minutes and the supernatant solutions decanted and discarded. Five ml of DMSO were added to each sample, the tubes covered with aluminum foil and heated for one hour in an Arnold sterilizer and cooled. The sterol was extracted with three 10 ml volumes of n-hexane and the extracts evaporated to dryness under N_2. Sterol content of the extracts was determined by Liebermann-Burchard color.

Mutant yeast culture

Yeast mutant strain KD 46, which requires either ergosterol or oleic acid for growth (69), was cultured on agar plates of Wickerham's complete medium and on plates containing Wickerham's complete medium to which column fractions containing 50 μg/ml. ergosterol had been added to give a final concentration of 12.5 μg/ml. Additional plates of Wickerham's complete medium containing an equal amount of complexing agent to which no ergosterol had been added were also utilized. All cultures were incubated at 30°C and the time of appearance of colonies was noted. Purity of the resultant growth was determined by microscopic examination.

Mutagenesis with ultraviolet irradiation

Strain 3701 B was inoculated from a YCM agar slant into WCY media and aerated on a reciprocal shaker at 30°C for 24 hours. The culture was then centrifuged, washed with sterile distilled water and reinoculated into tubes of WCY medium at 1/10 concentration. This culture was then incubated for six hours, centrifuged washed and resuspended in 50 ml of phosphate buffer, pH 6.6. Ten ml of this suspension were then placed in a sterile petri dish. The plate was swirled on a rotary shaker and exposed to ultraviolet light of an energy dose of 12 ergs/mm^2/sec for periods of from 45 to 75 seconds. Samples of from 0.2 to 0.5 ml of the irradiated

suspension were then transferred to tubes containing WCY medium containing ergosterol at 5 μg/ml added as an ethanolic solution prior to autoclaving of the medium. Inoculation of the culture was performed under a red light to protect the cells from photoreactivation. The cultures were then aerated in the dark for 16 to 24 hours, centrifuged, washed, the cell concentration determined with a cell counting chamber and the suspension spread to 75 to 100 cells per plate on YCM agar with 100 μg of ergosterol spread on the surface of the plates in ethanolic solution. The alcohol was allowed to evaporate from the surface of the plates before plating of the cells. Plates were then incubated until individual colonies had reached three to five mm in diameter. For detection of mutants the replica plating technique of Lederberg and Lederberg(43) was employed. The plates were stamped and transferred via velveteen pads to Wickerham's minimal agar, Wickerham's complete agar, and TCA agar, with and without ergosterol supplements of 100 μg/plate spread on the surface of the agar. The plates were incubated at 30°C and observed for mutant colonies.

During the course of attempting to isolate a sterol mutant, minor variations of the above procedures were employed. Instead of using commercial ergosterol, a mixture of yeast sterols isolated from Fleischmann's yeast was employed at a concentration of 25 μg/plate or included in the agar medium at concentrations of from

one to three μg/ml.

Mutagenesis with nitrous acid

When nitrous acid was employed as a mutagen, strain 3701 B was inoculated into WCY medium and aerated for 25 hours. This culture was washed and resuspended in 20 ml of 0.5 M sodium acetate buffer (pH 4.7) and then added to 20 ml of a 0.4 M solution of nitrous acid and incubated at 30°C. Samples were removed at intervals up to 12 minutes and immediately diluted one to ten in a solution of 1% yeast extract and 2.7% Na_2HPO_4. One ml volumes of this suspension were then inoculated into WCY broth containing 2 μg/ml ergosterol and aerated at 30°C. Mutants were assayed by the replica plating technique previously described.

Mutagenesis with ultraviolet irradiation with sterol supplied as the water-soluble complex

The isolation of a soluble complex of ergosterol from yeast extract prompted a renewed effort to isolate a sterol mutant using the complex as a solubilizing agent. A mixture of yeast sterols isolated from Fleischmann's dry yeast was solubilized by combining with yeast extract and passing through Sephadex G-50 columns. Column fractions were lyophillized, their sterol content determined by treatment with DMSO, and added to defined media prior to autoclaving. The preparation and irradiation of the

cultures was performed as previously described. The irradiated cultures were inoculated into YCC broth containing 12 μg/ml sterol added as ethanolic solution prior to autoclaving the media and grown at 30°C for 16 hours either statically or on a reciprocal shaker. The cultures were then washed and spread onto YCC agar plates containing 12 μg/ml sterol supplements. Sterol was added to the defined medium as the soluble complex.

RESULTS

It had been observed that ethanolic solutions of ergosterol added to culture media resulted in an inability to re-extract the sterol with petroleum ether. A series of experiments was designed to test which constituents of the medium effected the binding. The results are shown in Table 3. It may be seen that as much as 94% of the proferred sterol became resistant to solvent extraction from the YCM medium and that almost all of this binding could be accounted for by the presence of yeast extract.

In order to investigate the binding of sterols by yeast extract, increasing quantities of ergosterol and cholesterol were added to 10 ml volumes of 5% yeast extract, the tubes mixed, autoclaved, and extracted with three 10 ml volumes of petroleum ether. The amounts of sterol recovered are shown in Table 4 and demonstrate the capacity of yeast extract to bind substantial quantities of both ergosterol and cholesterol.

In order to determine whether or not the amino acids present in the growth media were responsible for the binding of sterol, the binding capacity of a 3% solution of vitamin free Cas-amino acids (Difco Laboratories) was determined. The results demonstrated nearly quantitative recovery of the sterol. The Cas-amino acids bind less than 6% of the supplied sterol.

Table 3. Binding of Ergosterol by Constituents of Yeast Growth Media

Flask contents	1 μg Sterol recovered	2 μg Sterol recovered
YCM medium	12	32
2% glucose	126	143
2% Tryptone	75	65
1% yeast extract	18	35
Distilled H_2O	200	188
Phosphate buffer	179	182
Phosphate buffer Plus 2% glucose	182	185
Wickerham's Medium	143	144

Each flask contained 100 ml of the indicated solution to which was added 200 μg of ergosterol in 2.0 ml of ethanol. Columns 1 and 2 differ in that ergosterol was added prior to and subsequent to autoclaving, respectively. Sterol was extracted with successive 40, 20, 20, and 10 ml volumes of petroleum ether, B.P. 30° - 60°. Phosphate buffer consisted of 6.53 g K_2HPO_4 and 8.51 g KH_2PO_4 per liter to give a pH of 6.6.

Table 4. Binding of Ergosterol and Cholesterol by Solutions of Five Percent Yeast Extract

Sample	μg Sterol added	μg recovered*	μg Bound	% Bound
Ergosterol-1	100	8	92	92
Ergosterol-2	250	3	247	99
Ergosterol-3	400	13	387	97
Ergosterol-4	500	16	484	97
Cholesterol-1	100	4	96	96
Cholesterol-2	250	13	237	95
Cholesterol-3	400	17	383	96
Cholesterol-4	500	23	477	96

* Corrected for sterol of a yeast extract blank.

It was of interest to determine whether or not the added sterol could be recovered by extraction of the unbound sterol and release of the bound sterol to free sterol by means of acid and base hydrolysis. The results of such an experiment are given in Table 5 and demonstrate that in excess of 95% of the added sterol can be recovered by extraction and methanolic pyrogallol saponification.

That a water-soluble ergosterol complex is formed with yeast extract and that it is not dialyzable through ordinary dialysis membranes was shown as follows. A 5% w/v solution of yeast extract to which ergosterol had been added to a final concentration of 50 μg/ml was extracted with petroleum ether to remove uncombined sterol. The resulting aqueous solution was extensively dialyzed against 0.1M phosphate buffer and then lyophillized. The dried powder was redissolved in a small volume of water, subjected to pyrogallol saponification and extracted with petroleum ether. The Liebermann-Burchard color test and thin layer chromatography showed that the extract contained sterol. There was no significant loss of sterol through the dialysis membrane. The complexing material appeared to be of large molecular weight, since it could not pass through the membrane.

When sterol complex from Sephadex column fractions was applied to thin layer plates which were then developed in benzene-ethyl acetate 5:1, the carrier component remained at the origin

while the ergosterol separated as the free sterol. These results indicated that the binding of the sterol was noncovalent and suggested the possibility of separating the sterol from the complexing agent with the aid of digitonin. Table 6 shows that the sterol can indeed be precipitated from the complex with digitonin. The addition of the ethanolic digitonin solution, however, introduces a complication in that too much alcohol precipitates the complex and this makes the estimation of sterol in aqueous solutions of complex where the amount is unknown less satisfactory.

Ergosterol digitonide, when similarly applied to thin-layer plates after exhaustive washing with acetone-diethyl ether 1:1 to remove free sterol, also gives rise to a free sterol spot, but in this case some sterol also remains at the origin. This may mean that the noncovalent binding of ergosterol to digitonin is "stronger" than to the yeast component, or it may merely reflect a lower solubility of the digitonide in the developing solvent, benzene-ethyl acetate 5:1.

It was determined that the complex was precipitated with 50% alcohol and could be easily redissolved in water. Repeated precipitations with alcohol, however, resulted in the formation of a gummy precipitate which was difficult to resuspend in aqueous solution.

The complex in solution gave a red coloration when treated with iodine solution. When applied to the surface of thin layer plates and

Table 5. Recovery of Sterol from Yeast Extract by Extraction and Hydrolysis

Sample	Hydrolysis method	μg From wash	μg From hydrolysis	Total μg recovered
1	Acid	50.5	95.5	146
2	Base	50.5	40.5	91
3	Pyrogallol	50.5	408.0	458.5

Six tubes containing 10 ml volumes of 5% yeast extract to which had been added 500 μg quantities of ergosterol were extracted with three 10 ml volumes of petroleum ether and the sterol content of the extracts determined. The tube contents were then hydrolysed by the three methods described below and the extractable sterol content determined. The average value is reported here.

1) 10 ml of 0.2 N HCl were added, the tubes heated in an Arnold sterilizer for one hour, the pH adjusted to 10, and the tubes extracted.

2) 10 ml of 40% KOH were added, the tubes heated for six hours in an Arnold sterilizer and extracted.

3) 3.0 ml of 0.5% pyrogallol in absolute methanol, 2.0 ml of 60% KOH and 3.0 ml of absolute methanol were added and the tubes refluxed with cold fingers for one hour and extracted.

Table 6. Digitonin Precipitation of Ergosterol Out of the Yeast Complex

Conditions		Yield of	
Complex*	Digitonin	sterol	Ratio sterol:complex
ml	ml	μg	μg/ml
3.0	1.0	190	63
3.0	1.0	190	63
3.0	2.0	194	65
3.0	2.0	199	67
5.0	2.5	291	58
5.0	2.5	366	53

* "Complex" refers to Sephadex column fraction of yeast extract to which ergosterol has been added.

dried, the complex gave a negative ninhydrin reaction but gave an intensely positive reaction when sprayed with periodate and then benzidine. The presence of a compound with vicinal hydroxyl groups is indicated.

Lyophillized hot water extracts of whole dry yeast were assayed to determine if the sterol complex could be isolated from that source. A 15% w/v solution of this extract was compared to a similar solution of Difco yeast extract to which 150 μg/ml ergosterol had been added. Aliquots of the solutions were analyzed for sterol prior to gel filtration, the fractions from which were also analyzed for sterol by three different procedures. The results of these analyses are given in Table 7 and demonstrate that the sterol is in fact present in the alcohol precipitable fractions from the columns and that a soluble form of sterol occurs naturally in yeast and can be isolated from hot water extracts of yeast.

The ability of DMSO to cleave sterol from the complex was investigated because it has this action of digitonides (33). The data in Table 8 show that sterol is extracted by hexane from the DMSO treated complex in a yield directly proportional to the initial amount of complex. This technique was used for determining the amount of ergosterol present in the solutions of complex isolated from a Sephadex G-50 column. The elution profile is presented in Figure 3 and shows that the sterol is eluted immediately after the void volume

Table 7. Recovery of Sterol From Yeast Extract Complex

Sample	Hydrolysis method	Sterol recovered
		μg
1A	Pyrogallol saponification	86
1B	Pyrogallol saponification	201
2A	Acid hydrolysis	21
2B	Acid hydrolysis	98
3A	Pyrogallol saponification	60
3B	Pyrogallol saponification	223
4A	Pyrogallol acid hydrolysis	36
4B	Pyrogallol acid hydrolysis	41

A and B, respectively, refer to hot water extracts of whole yeast and to commercial yeast extract to which ergosterol had been added. Samples 1A and 1B were the untreated yeast extract preparations while the remaining samples were corresponding preparations that had been subjected to column chromatography for purification of the ergosterol complex.

Table 8. Extraction of Sterol From the Complex By Means of DMSO

Complex	Yield of sterol*
ml	μg
2.0	112± 3
3.0	154± 5
4.0	197± 3
5.0	241± 5

* Mean of duplicate determinations and actual range.

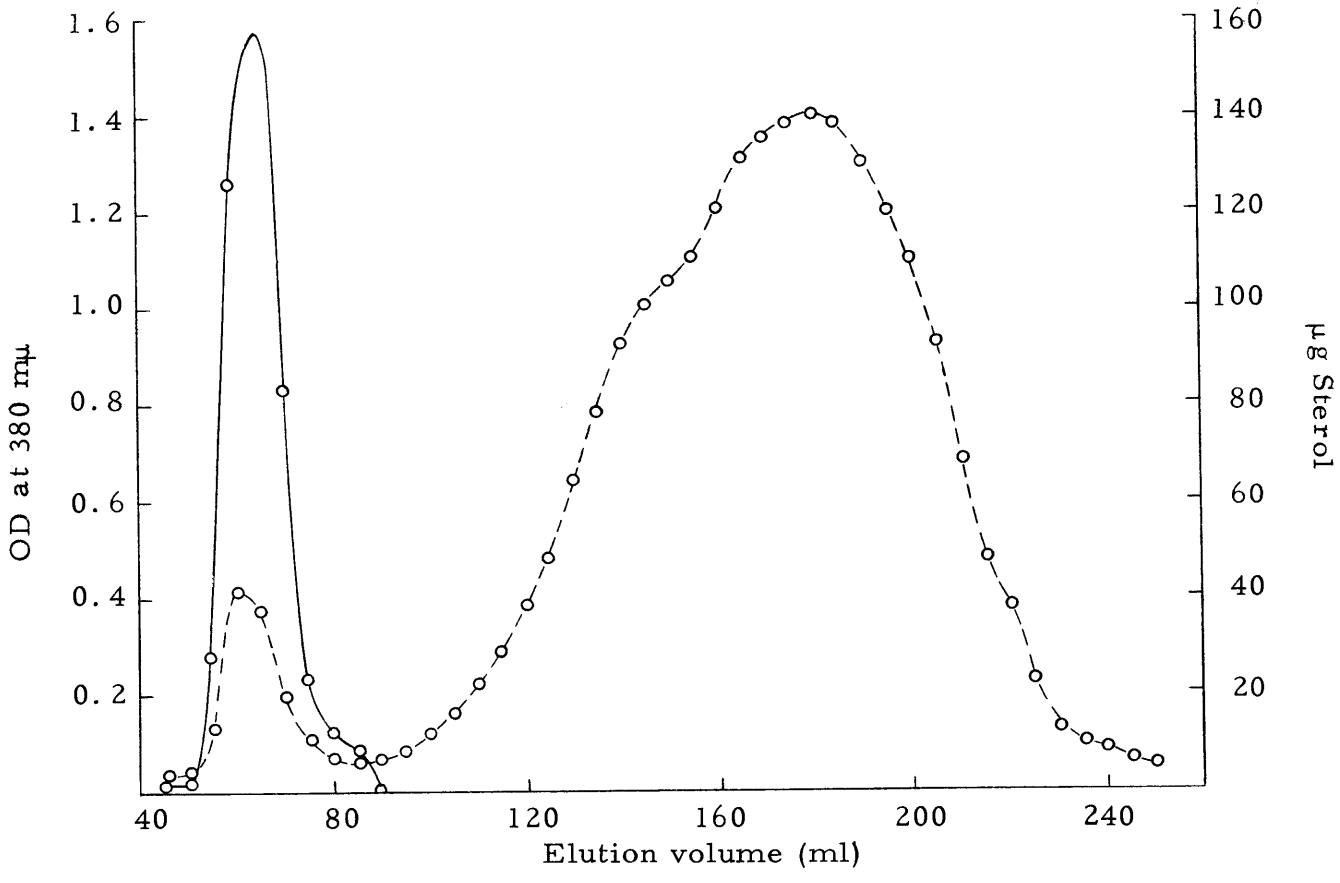


Figure 3. Chromatography of a 12 ml solution of 15% yeast extract containing 150 μg/ml ergosterol on a Pharmacia glass column, 2.5 cm I.D., packed to a heighth of 31 cm with Sephadex G-50. The solid line represents amount of sterol obtained by the DMSO procedure; the broken line represents nonspecific absorbancy at 380 mμ.

of the column without appreciable tailing and is separated from the bulk of the small molecular weight components contained in the yeast extract.

The ultraviolet absorption spectrum of ergosterol in the complex was determined on column fractions, first diluted four times with distilled water. The spectrum exhibited shoulders at 282 and 297 mμ and peaks at 262 and 271 mμ. These are similar to literature values for ergosterol (26, p. 93) and to curves obtained on the same instrument with ergosterol in methanol showing a shoulder at 262 mμ and peaks at 271, 282, and 294 mμ.

Metabolic availability of ergosterol in the soluble complex was demonstrated by the use of the yeast mutant strain KD 46. This organism requires either ergosterol or oleic acid for growth (69) and was obtained from Drs. M. A. Resnick and R. K. Mortimer. Cells of this strain were cultured on Wickerham's defined media with and without sterol complex supplements as previously described. After four days incubation colonies appeared on the medium supplements with ergosterol complex; no growth at all was observed in the controls, even after two weeks incubation. Thus, the ergosterol complex is able to provide the ergosterol required for growth by this mutant.

In order to determine the amount of sterol contained in lyophillized sterol complex and in the complexing agent, 55 ml volumes

of 15% yeast extract to which ergosterol or cholesterol had been added to 150 μg/ml and 55 ml of 15% extract with no sterol added were passed through the large Sephadex G-50 columns. The first 60 ml of eluent followed the void volume of the columns were collected and lyophillized. Duplicate 100 and 200 mg amounts of ergosterol and cholesterol complexes and duplicate 200 mg amounts of the complexing agent were then dissolved in five ml of DMSO, heated 30 minutes in an Arnold sterilizer, cooled and extracted with three 10 ml volumes of petroleum ether. The extracts were evaporated and their sterol content determined by Liebermann-Burchard color against ergosterol and cholesterol standards. The results of these analyses are shown in Table 9.

It was of interest to examine more closely the chemical nature of the sterol complexing component from yeast extract. Complexing agent was isolated from 15% yeast extract by passage through Sephadex G-50 columns as described above. The volume of eluent taken was limited to 60 ml following the void volume of the column in order to avoid contamination with the small molecular weight components of the yeast extract.

Since the periodate-benzidine test, the iodine color reaction, and the precipitation with alcohol suggested the complexing agent might be a polysaccharide, solutions of the complexing agent were compared to solutions of glucose and of glycogen for their reaction

in the anthrone color test. Increasing amounts of a 1.0 mg/ml solution of complexing agent and of 200 μg/ml solutions of glucose and glycogen were added to sample tubes and brought to 1.0 ml volume with distilled water. Four (4.0) ml of a solution of 0.2% anthrone in concentrated H_2SO4 were added to each sample and the samples immediately mixed and read at 625 mμ on a Beckman model DU spectrophotometer. The results of these determinations are shown in Table 10.

The amount of protein present in the complexing agent was investigated using the colorimetric determination of Lowry and co-workers (46) against bovine serum albumin standards. Color was read on a Beckman model DU spectrophotometer at 625 mμ. The samples of complexing agent up to 1.0 mg/ml were found to be completely negative for Lowry protein color.

Since it appeared that the complexing agent was some kind of a polysaccharide, an effort was made to identify the component carbohydrate residues. One hundred mg of lyophillized complexing agent were dissolved in 10 ml of 1N H_2SO_4 in a tube fitted with a condenser and relfuxed at 105°C for 2.5 hours. The hydrolysate was cooled and neutralized by the addition of $BaCO_3$. To the neutralized solution 2.0 g of Bio Rad Ag 11A8 (50-100 mesh) analytical grade ion retardation resin (Calbiochem Company) were added, the tube shaken, centrifuged and the supernatant liquid decanted and filtered. Thin

Table 9. Extraction of Sterol From Lyophillized Ergosterol and Lyophillized Cholesterol Complex By Means of DMSO

Sample	Yield of sterol*
	μg/mg dry weight
Complexing agent 200 mg	0.28± 0.01
Ergosterol complex 100 mg	2.32± 0.06
Ergosterol complex 200 mg	2.02± 0.03
Cholesterol complex 100 mg	2.18± 0.16
Cholesterol complex 200 mg	1.74± 0.05

* Mean of two determinations and actual range.

Table 10. Anthrone Color Test on Glucose, Glycogen and Complexing Agent

Sample	μg	μg/O.D. unit	Average
Complexing agent	100	658	
Complexing agent	200	709	
Complexing agent	300	723	
Complexing agent	400	704	
Complexing agent	500	690	726
Complexing agent	600	750	
Complexing agent	700	757	
Complexing agent	800	755	
Complexing agent	900	796	
Complexing agent	1000	719	
Glucose	20	68	
Glucose	50	74	
Glucose	100	72	74
Glucose	150	83	
Glucose	200	*	
Glycogen	20	64	
Glycogen	50	80	
Glycogen	100	83	78
Glycogen	150	83	
Glycogen	200	*	

* O.D. too high to be accurate.

layer chromatography was performed on Silica Gel G plates as previously described. Several solvent systems were employed. When it became clear that the hydrolysate appeared to consist of a single component, a solvent system capable of separating glucose from galactose and mannose was employed. This system consisted of n-butanol:isopropanol:H_2O 5:3:1 (32, p. 600). Following development in this system the plates were sprayed with Petek's reagent (32, p. 582). Following heating for five minutes at 110°C this reagent causes fructose and fructose containing oligosaccharides to turn yellow, aldohexoses to turn brown, aldopentoses to turn pink and uronic acids to turn red. The plates were also sprayed with orcinol reagent for the detection of pentoses (6, p. 102). Samples of the hydrolysate and of glucose, galactose and fructose were run in this system and the Rfs and rg x 100s determined. The results are given in Table 11. The hydrolysate gave a single spot upon charring with 50% H_2SO_4, a brown coloration with Petek's reagent, and a negative orcinal reaction for pentoses. It would appear that the complexing agent consists solely of glucose subunits.

Considering the evidence that the complexing agent was a polysaccharide and the fact that the major carbohydrate reserve in yeast is glycogen (21), the ability of glycogen and trehalose, another yeast carbohydrate reserve, to bind ergosterol was investigated. Ergosterol was added to solutions of glycogen and trehalose dihydrate

and extracted with petroleum ether as previously described. The results of several such determinations are summarized in Table 12 and demonstrate that both glycogen and trehalose exhibit a limited and variable capacity to bind exogenous ergosterol. In order to determine if the binding of ergosterol by glycogen was similar to that of the complexing agent isolated from yeast extract, the ability of DMSO to cleave sterol from the glycogen-ergosterol complex was determined. A solution of 1% glycogen to which ergosterol had been added to 50 μg/ml was distributed in increasing volumes in duplicate centrifuge tubes. Equal volumes of 95% ethanol were added and the tubes stored at -20°C overnight, centrifuged, the supernatant liquid decanted and discarded, and 5.0 ml of DMSO were added. The tubes were heated for 30 minutes in an Arnold sterilizer, cooled, extracted, and the sterol determined by Liebermann-Burchard color. The results of this determination are shown in Table 13.

It was of interest to determine if yeast extract was capable of binding nonsterolic compounds. Since some of the compounds to be tested were extremely volatile and would be lost upon evaporation of the solvent, it was decided that ^{14}C labeled compounds would be used for quantitation of results. Instead of using the usual extraction procedure involving three extractions with 10 ml volumes of petroleum ether, a method termed solvent partition was employed. This method consisted of adding the compound of known specific activity

Table 11. TLC of Complexing Agent Hydrolysate in N-Butanol: Isopropanol: Water

Component	Rg x 100*	Rf*
Fructose	88	0.485
Galactose	83	0.459
Glucose	100	0.552
Hydrolysate	101	0.560

* Mean of distance traveled by 10 μg and 20 μg quantities.

Table 12. Binding of Ergosterol by Glycogen and Trehalose Dihydrate

Sample	μg Sterol added	μg Bound	% Bound	Range %
0.5% Glycogen	250	150	60	23 - 80
0.5% Glycogen	500	324	65	7 - 87
1% Glycogen	250	163	65	40 - 84
1% Glycogen	500	212	57	13 - 79
1% Glycogen	1000	300	30	23 - 38
0.5% Trehalose	250	138	55	37 - 71
0.5% Trehalose	500	144	29	0 - 56
1% Trehalose	250	109	44	35 - 59
1% Trehalose	500	148	30	7 - 51
H_2O	250	78	31	21 - 41
H_2O	300	140	47	43 - 51

Values for glycogen are the average of six determinations; values for trehalose are the average of four determinations; values for water are the average of two determinations.

to be tested to 10 ml volumes of yeast extract, adding exactly 10 ml of petroleum ether to the tubes and shaking the tubes for 30 seconds. From the supernatant solvent 5.0 ml volumes were removed from each sample to 15 ml of 4/3 strength scintillation fluid. The extracts were then counted against ^{14}C-toluene standards in 5.0 ml of petroleum ether and 15.0 ml of 4/3 strength scintillation fluid. The amount of the compound bound by the yeast extract was determined from these counts obtained and the determined specific activity of the labeled compound added. The results of such determinations showing the binding of hexadecane-1-^{14}C by 15% yeast extract and the binding of ^{14}C-pulegone and ^{14}C-squalene by 5% yeast extract are shown in Tables 14, 15, and 16, respectively. From the data, one can conclude that whereas substantial amounts of hexadecane are bound, the binding of pulegone is negligible. The results of the squalene determination were interesting in that the recovery of activity from the H_2O controls was so low. An analysis of the aqueous portions of the samples failed to account for the large majority of the activity. It was observed, however, that the squalene appeared to form an oily film at the interface between the aqueous and solvent layers of the samples.

The decline in sterol content coinciding with the exhaustion of glucose from the medium in aerated nonprofilferating cell suspensions reported by Parks and Starr (64) suggested the possibility that

Table 13. Cleavage of Ergosterol From the Glycogen-Ergosterol Complex with DMSO

Volume of solution	Yield of sterol*
ml	μg/ml
3	43± 4
5	44± 2
7	45± 0
10	47± 1

* Mean of two determinations and actual range.

Table 14. Binding of Hexadecane by 15 Percent Yeast Extract Measured by Solvent Partition

Sample	mg Added	mg Recovered*	mg Bound	% Recovered	% Bound
H_2O Blank	2	1.49	0.51	75	25
H_2O Blank	5	3.89	1.11	78	22
YE-1	1	0.21	0.79	21	79
YE-2	2	0.23	1.77	12	88
YE-3	3	0.29	2.71	10	90
YE-4	4	0.33	3.67	8	92
YE-5	5	0.26	4.74	5	95

* Mean of two determinations corrected by a factor of two.

Table 15. Binding of Pulegone by Five Percent Yeast Extract Measured by Solvent Partition

Sample	µg Added	µg Recovered*	% Recovered
H_2O Blank	100	100	100
H_2O Blank	400	390	98
YE-1	100	99	99
YE-2	200	196	98
YE-3	300	289	97
YE-4	400	382	96
YE-5	500	479	96

* Mean of two determinations corrected by a factor of two.

Table 16. Binding of Squalene by Five Percent Yeast Extract Measured by Solvent Partition

Sample	µg Added	µg Recovered*	µg Bound	% Recovered	% Bound
H_2O Blank	200	24	176	12	88
H_2O Blank	500	29	471	6	94
YE-1	100	17	83	17	83
YE-2	200	15	185	8	92
YE-3	300	17	283	6	94
YE-4	400	20	380	5	95
YE-5	500	24	476	5	95

* Mean of two determinations corrected by a factor of two. One ml volumes of the 500 µg samples of the H_2O and yeast extract solutions were counted using thixcin gel after extraction. The H_2O sample counts revealed the aqueous phase to contain but 23% of the activity and the aqueous phase of the yeast extract sample to contain but 35% of the total activity. Total recovery of the two phases was thus 29% for the H_2O sample and 40% for the yeast extract sample.

the sterol, rather than being degraded, was being converted from an alkali hydrolysable form to some type of nonalkali hydrolysable form as the cells started to respire using the accumulated ethanol as substrate. The classical method of recovering total sterol from cells has been to saponify the cells and extract the nonsaponifiable fraction with organic solvents. This method is based on the premise that the sterol exists either as free sterol or as fatty acid sterol esters. However, if the sterol exists in yet another form, refractory to saponification, then methods of treatment other than saponification may be capable of recovering additional sterol. With this in mind, investigations were carried out to determine if additional sterol could be obtained from yeast using acid hydrolysis.

Yeast cells grown microaerobically were resuspended in aeration medium. Following aeration on a rotary shaker for two hours, five μc of methyl-^{14}C-methionine were added and the cells aerated an additional five hours. The cultures were then centrifuged, washed three times with cold distilled water, resuspended in water and distributed in five ml volumes in six sets of triplicate extraction tubes and treated by one of the following procedures:

1) The cells were treated with routine saponification.

2) 5.0 ml of 0.1N HCL were added to each tube and the tubes were incubated for one hour at 30^{o}C; the pH was adjusted to 10 and the tubes extracted.

3) 5.0 ml of 0.1N HCl were added to each tube, and the tubes placed in an Arnold sterilizer for one hour; the pH was adjusted to 10 and the tubes extracted.

4) 5.0 ml of 0.1N HCl were added to each tube, the tubes incubated for one hour at 30°C and centrifuged; the supernatant liquid was decanted and discarded, the volume brought +05.0 ml with water after which 5.0 ml of 40% KOH were added; the cells were then placed in an Arnold sterilizer for six hours, cooled, and the tubes extracted.

5) To each tube 2.5 ml of 0.1N HCl and 2.5 ml of 95% ethanol were added. The tubes were refluxed for one hour with cold fingers, cooled, the pH adjusted to 10 and the tubes extracted.

6) 5.0 ml of H_2O were added to each tube; the tubes were placed in an Arnold sterilizer for one hour, cooled, and extracted.

In all cases the petroleum ether extracts were evaporated to dryness under nitrogen in scintillation vials, fluid added and the radioactivity determined. The results of the triplicate analyses are shown in Table 17, values being expressed as a percentage of those obtained with routine saponification. From these data it is obvious that the method of routine saponification used is not releasing a considerable amount of sterol present in the cells.

In order to investigate the relationship of acid hydrolysable sterol to that of alkali hydrolysable sterol and to determine the

Table 17. Extraction of Sterol by Acid Hydrolysis

Procedure	Counts per minute*	Percentage of saponification
1. Saponification	41,667	100.0
2. Cold acid	14,837	35.6
3. Hot acid	72,028	172.9
4. Cold acid and saponification	67,578	162.2
5. Acid-alcohol reflux	72,860	174.9
6. Hot water	16,657	40.0

* Each value is the average of triplicate determinations.

effect of a slowly fermented substrate such as galactose in microaerobically grown cultures on subsequent sterol synthesis, the following series of experiments was carried out.

Cells of strain 22 B were grown microaerobically in YMAF medium employing 5% glucose and 5% galactose for 40 hours at 30°C. The cells were allowed to settle in the cold, centrifuged, washed four times with cold 0.1 M phosphate buffer and resuspended in 100 ml of aeration medium. To each flask five μc of methyl-^{14}C-methionine were added and the flasks aerated on a rotary shaker at 30°C. At intervals duplicate five ml aliquots were removed from each flask and washed with two 5 ml volumes of ice cold distilled water. The cells were then lyophillized, the dry weight determined, the cells then resuspended in a small volume of distilled water. One of the duplicate samples from each flask was treated with routine

saponification, the other sample was subjected to hot acid treatment. The pH was then adjusted to 10, the tubes extracted, and activity determined as previously described. The data are plotted in Figures 4, 5, and 6. Sterol values are given as cpm/mg dry weight of cells. The results reported here are those obtained from a single experiment. It should be emphasized that these fluctuations have been observed in a large number of experiments of this type, although are occasionally displaced in time slightly earlier or later depending on the cell concentrations for that particular experiment.

An additional time course analysis was performed using cells grown microaerobically on 5% glucose as described above but which were subsequently aerated in aeration medium containing sodium acetate rather than glucose. The cells were washed and resuspended in an aeration medium consisting of 0.1M KH_2PO_4 and 2.3% sodium acetate. Ten μc of methyl-^{14}C-methionine were added to the flask and the suspension aerated on a rotary shaker at 30°C. At intervals duplicate samples were removed, washed, and lyophillized as previously described. The dry weight was determined and the cells saponified in the routine manner. The results of this analysis are presented in Figure 7.

Parks and Starr (64) have reported the effects of various respiratory inhibitors on the sterol content of cells harvested from aerated nonproliferating suspensions. With the exception of sodium

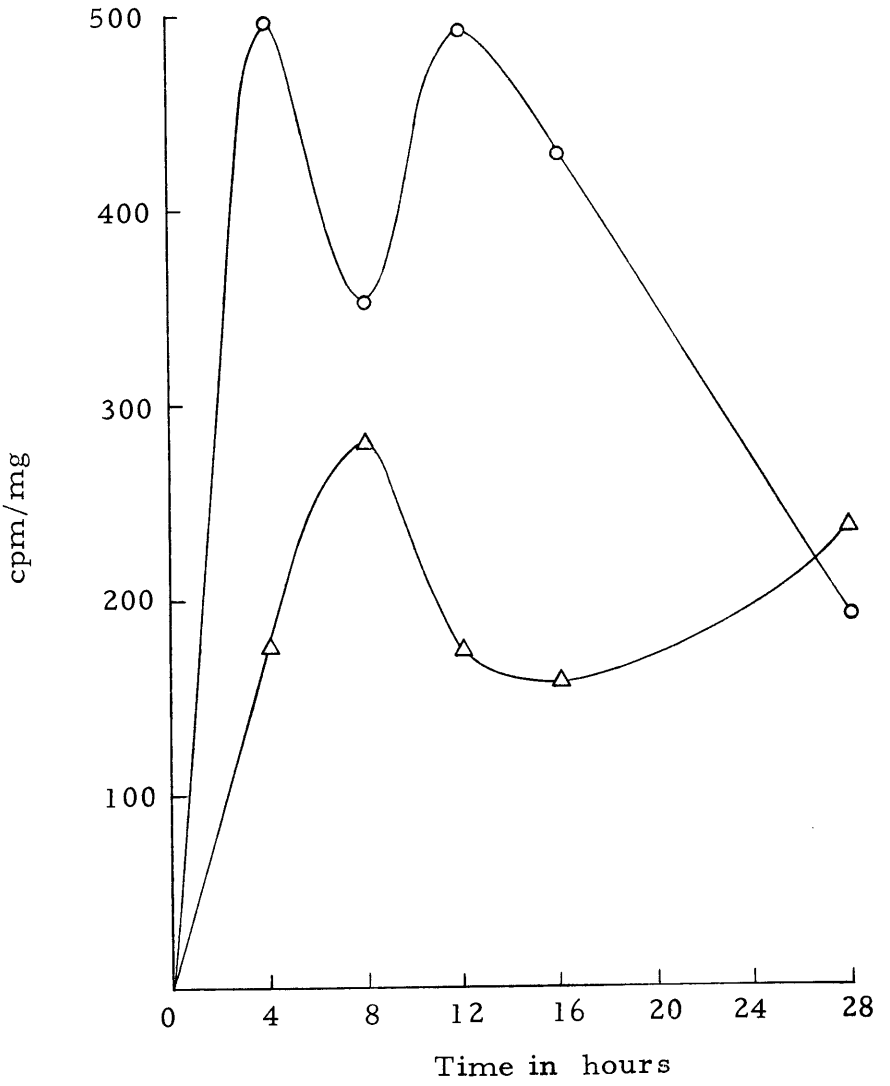


Figure 4. Time course analysis of sterol content of cells grown anaerobically on 5% glucose and aerated in aeration medium containing 1% glucose as carbon source. The circles indicate sterol recovered from cells following treatment with hot acid; the triangles indicate sterol recovered from cells following saponification.

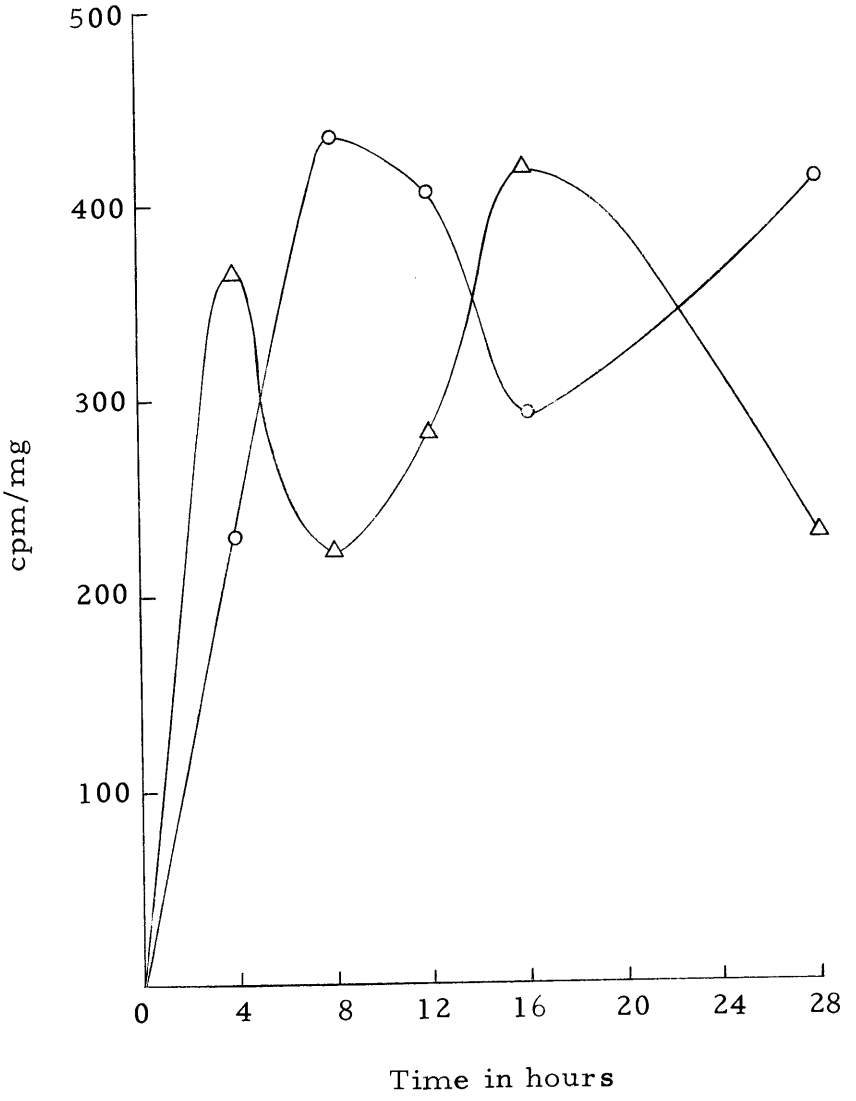


Figure 5. Time course analysis of sterol content of cells grown anaerobically on 5% galactose and aerated in aeration medium containing 1% glucose as a carbon source. The circles indicate sterol recovered from cells following treatment with hot acid; the triangles indicate sterol recovered from cells following saponification.

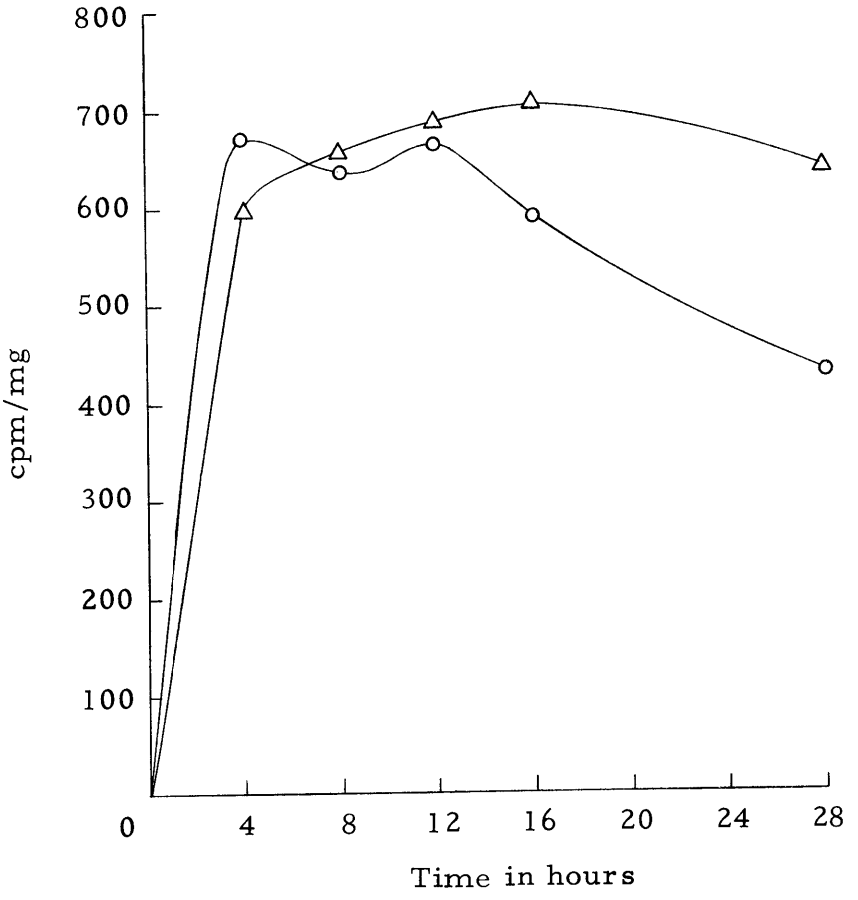


Figure 6. Summation of acid and base labile sterol recovered from glucose and galactose grown cells aerated in aeration medium containing 1% glucose as a carbon source. The circles indicate the summation from glucose grown cells; the triangles indicate the summation from galactose grown cells.

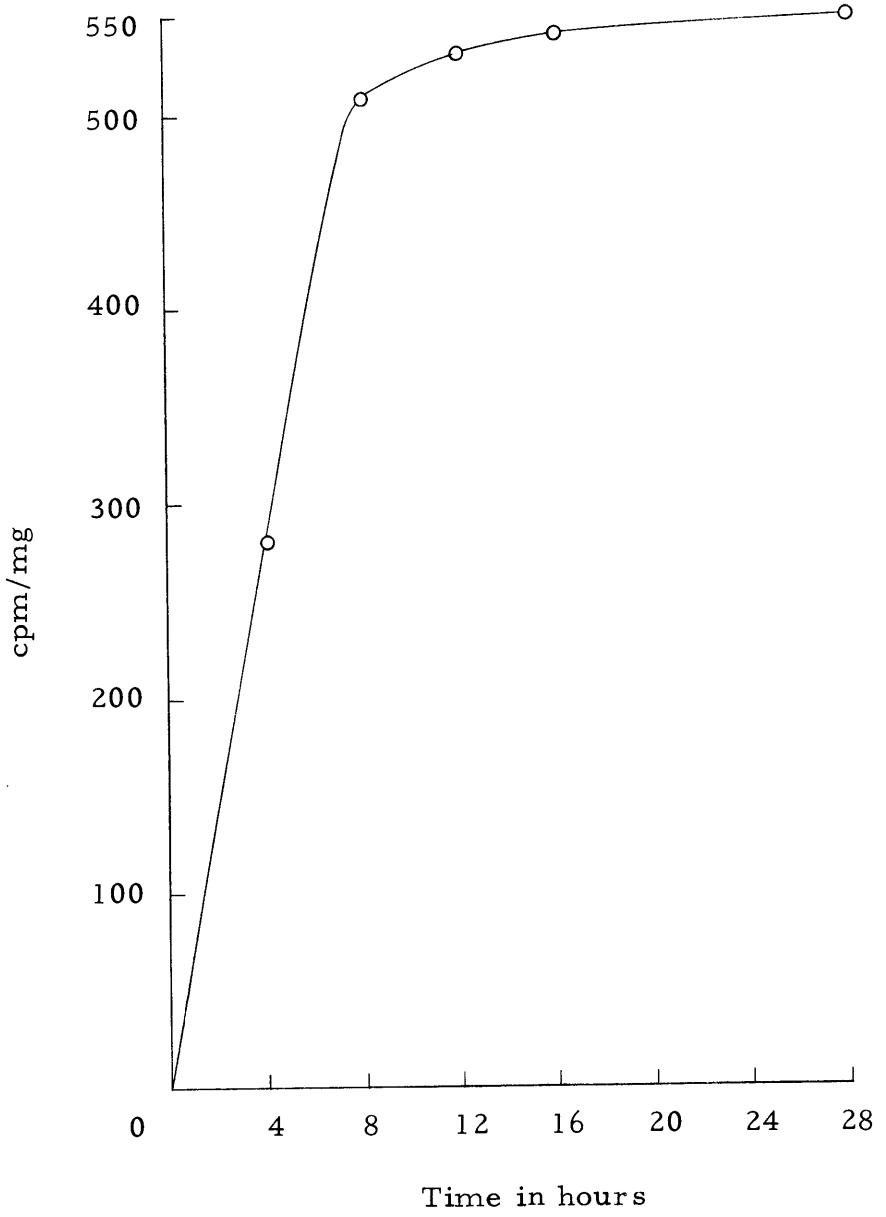


Figure 7. Time course analysis of sterol content of cells grown anaerobically on 5% glucose and aerated in aeration medium containing 2. 3% sodium acetate as a carbon source. The circles indicate the average values obtained from saponification of duplicate samples.

azide, these inhibitors caused a reduction in the sterol content of the cells. Since it would appear that there exists a relationship between sterol metabolism of cells and their respiratory state, an investigation of the effect on the time course sterol content of cells in aerated nonproliferating cultures was performed as follows.

Four 1800 ml flask of YMAF medium and four 1800 ml flasks of YMAF containing inhibitor were inoculated with strain 22 B and incubated statically for 48 hours at 30° C. The cells were allowed to settle in the cold, centrifuged, washed four times with ice cold phosphate buffer and each culture resuspended in 400 ml of cold buffer. The cell concentration of each suspension was determined with a Model F Coulter Counter using a 100 μ aperture and appropriate amounts of suspension removed to effect equal cells numbers in each flask. The suspensions were then divided into two equal parts and centrifuged. One-half of each pelleted suspension was resuspended in 200 ml of aeration medium and the other half in 200 ml of aeration medium containing inhibitor at the same concentration used in the growth medium. To each cell suspension 2.5 μc of methyl-^{14}C-methionine were added and the flasks aerated on a rotary shaker at 30°C. Duplicate five ml samples were removed at intervals and centrifuged, washed with two ml volumes of cold distilled water and resuspended in 1.0 ml of cold distilled water. Nine ml of 60% KOH-absolute methanol-0.5% pyrogallol in absolute

ethanol 3:3:3 were added to each sample and the samples refluxed for one hour, cooled and extracted. The extracts were evaporated in scintillation vials and counted against ^{14}C-toluene standards.

The inhibitors used and the concentrations employed are as follows: pentamethylenetetrazol (Metrazol) at 0.4 mM, sodium azide at 3.0 μg/ml and acriflavin at 1.5 μg/ml. The time course analyses using Metrazol, sodium azide and acriflavin are presented in Figures 8, 9, and 10, respectively.

It has been shown that chloramphenicol employed at 4 mg/ml in the growth medium inhibits the development of the components of the respiratory apparatus which are synthesized on the 70S ribosomal protein synthesizing system characteristic of the mitochondrion (12). Kovac and co-workers (41) have demonstrated that this same concentration of chloramphenicol included in a nitrogen free aeration medium results in a two-thirds reduction in the sterol content of that of control cells following eight hours of aeration. It was of interest to determine the time course sterol content of cells aerated in the presence of chloramphenicol. Additionally, since the specific activity of the methyl-^{14}C-methionine added to the suspensions was quite high (10 μc/uM) and the actual amount of methionine added thus rather low, the effect of cold methionine added after eight hours of aeration on subsequent labeled sterol content of the cells was also investigated as described below.

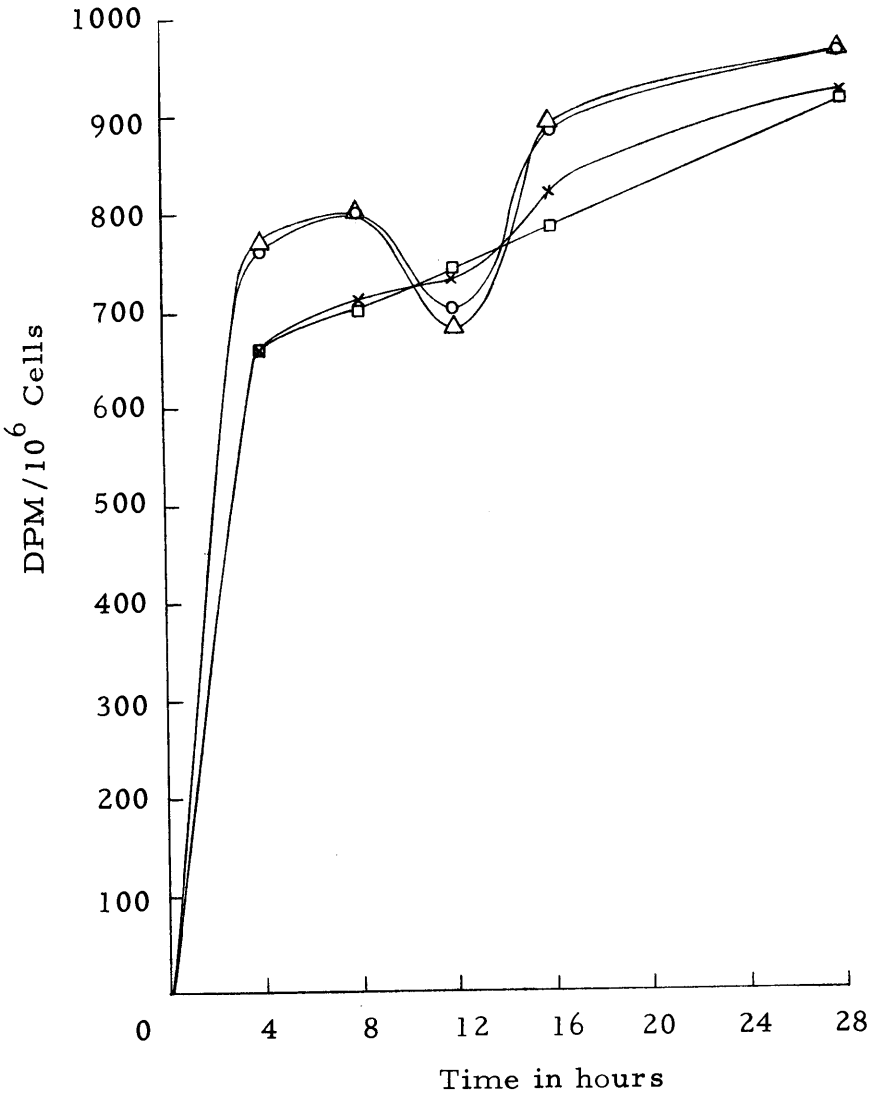


Figure 8. Time course analyses of cells aerated in aeration medium containing 1% glucose as a carbon source in the presence and absence of 0.4 mM Metrazol. The cells were previously grown microaerobically in the presence and absence of 0.4 mM Metrazol. The circles indicate cells grown and aerated in the presence of Metrazol; the squares indicate cells grown in the presence of Metrazol and aerated in its absence; the crosses indicate cells grown and aerated in the presence of Metrazol. The cell concentration was 3.67×10^8 cells/ml.

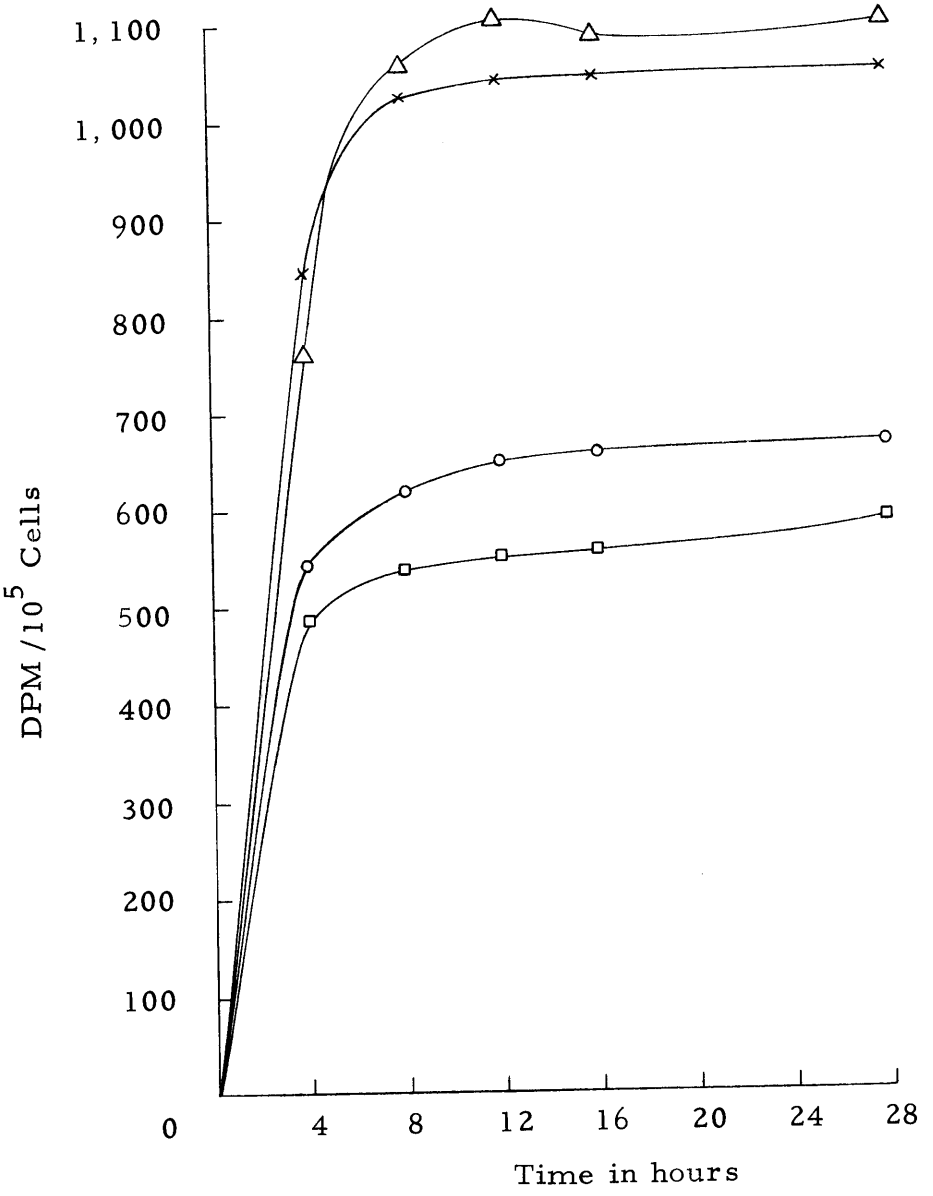


Figure 9. Time course analyses of cells aerated in aeration medium containing 1% glucose as a carbon source in the presence and absence of 3.0 μg/ml sodium azide. The cells were previously grown microaerobically in the presence and absence of 3.0 μg/ml sodium azide. The circles indicate cells grown and aerated in the absence of azide; the triangles indicate cells aerated in the presence of azide; the squares indicate cells grown in the presence of azide and aerated in its absence; the crosses indicate cells grown and aerated in the presence of azide. The cell concentration was 7.75×10^7 cells/ml.

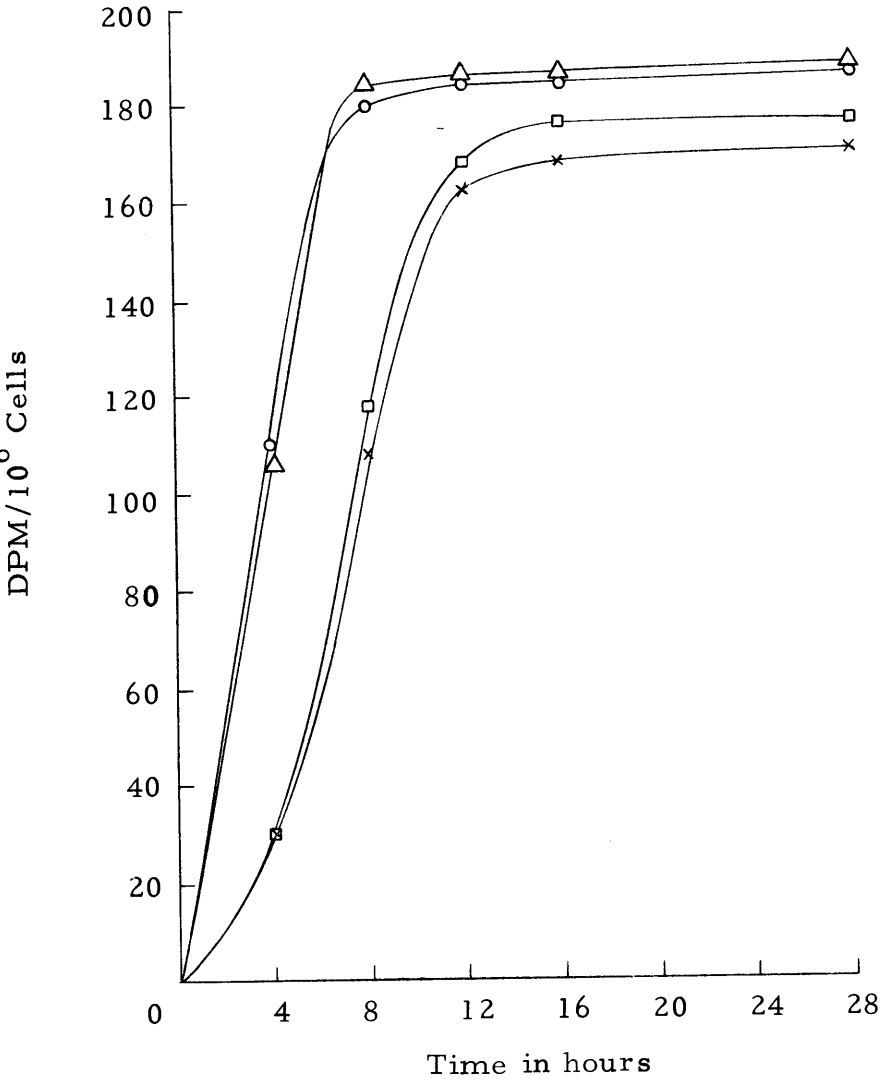


Figure 10. Time course analyses of cells aerated in aeration medium containing 1% glucose as a carbon source in the presence and absence of 1.5 μg/ml acriflavin. The cells were previously grown microaerobically in the presence and absence of 1.5 μg/ml acriflavin. The circles indicate cells grown and aerated in the absence of acriflavin; the triangles indicate cells aerated in the presence of acriflavin; the squares indicate cells grown in the presence of acriflavin and aerated in its absence; the crosses indicate cells grown and aerated in the presence of acriflavin. The cell concentration was 3.85×10^7 cells/ml.

Six flasks of YMAF cultures grown for 48 hours were harvested and washed as previously described. The cells were resuspended and divided into three equal parts and recentrifuged. Two of the cell pellets were resuspended in 200 ml volumes of aeration medium and the third in aeration medium containing 4 mg/ml chloramphenicol. To each flask 2.5 μc of methyl ^{14}C-methionine were added and the flasks aerated on a rotary shaker at 30^{o}C and samples taken at intervals. After eight hours of aeration 0.2 ml of a 5.0 mg/ml solution of cold methionine were added to one of the control flasks. The duplicate 5.0 ml samples were treated as previously described. The results of these analyses are shown in Figure 11.

In examining the time course data of labeled sterol content given in Figures 8, 9, 10, and 11, it can be seen that the previously described decline in sterol content of the cells occurs only in the control cells as presented in Figure 8. The control cells in Figures 9, 10, and 11 exhibit no such decline. An examination of the cell concentrations employed in the different experiments reveals that the concentration in the flasks for the Metrazol experiment is on the order of 10^8 cells/ml whereas those of the other experiments is on the order of 10^7 cells/ml. That the decline and subsequent increase in sterol content is detected only in cultures with the higher cell concentrations is supported by the data presented in Figure 12. In this experiment cells were grown and harvested as usual from

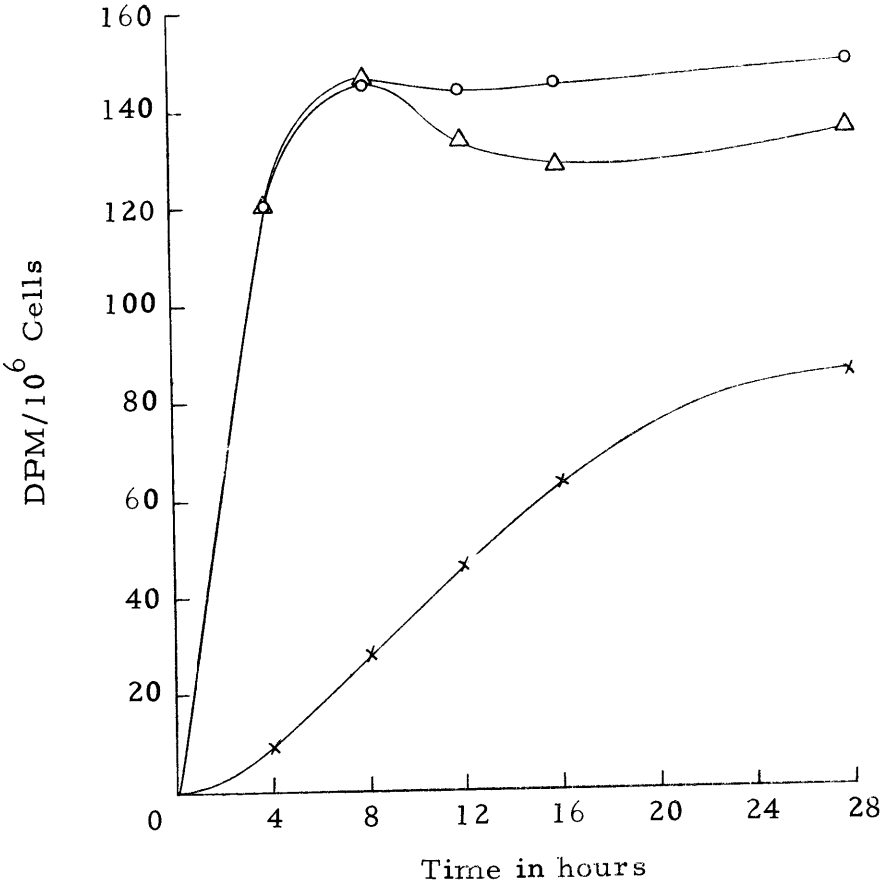


Figure 11. Time course analyses of cells aerated in the presence and absence of 4 mg/ml chloramphenicol and of cells following the addition of 5 μg/ml methionine after eight hours of aeration. The circles indicate cells aerated without supplements; the triangles indicate cells to which 5 μg/ml of methionine were added after eight hours of aeration; the crosses indicate cells aerated in the presence of 4 mg/ml chloramphenicol. The cell concentration was 5.65×10^7 cells/ml.

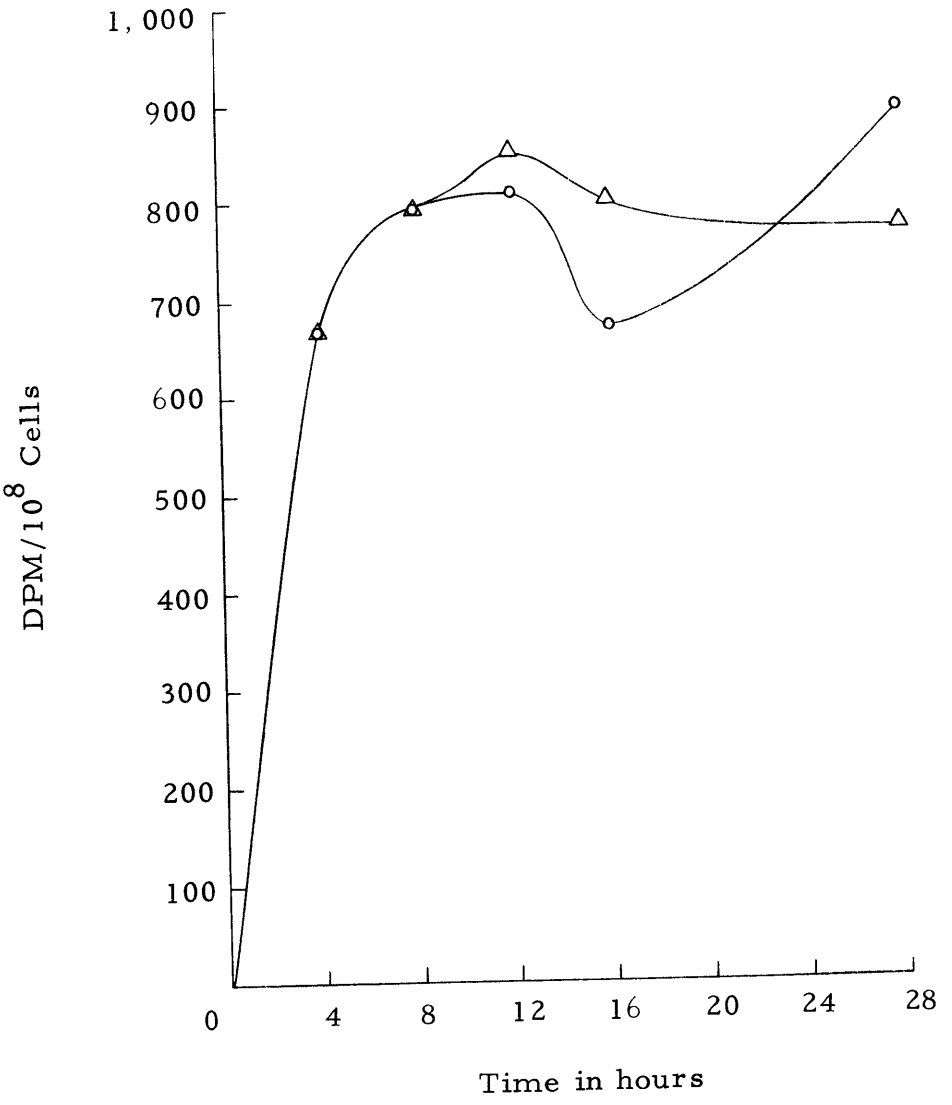


Figure 12. Time course analyses of cells aerated in the presence and absence of 1.5 μg/ml acriflavin. The circles indicate cells aerated in the absence of acriflavine; the triangles indicate cells aerated in the presence of acriflavine. The cell concentration was 5.32×10^8 cells/ml.

YMAF media and the cells from eight flasks resuspended in 400 ml of aeration medium. The suspension was then divided into two parts and 5 μc of methyl-^{14}C- methionine added to each part. To one of the suspensions acriflavine was added to a concentration of 1.5 μg/ml and the suspensions aerated on a rotary shaker at 30°C. At intervals duplicate 5.0 ml samples were taken and treated as previously described. The cell concentration determined with the Coulter counter was 5.32×10^8 cells/ml, and it can be seen from the graph that the control cells exhibit the decline and subsequent increase in sterol content.

In spite of repeated efforts, no strain of yeast exhibiting a permanent requirement for sterol could be isolated. Even though four to five percent of the colonies growing on Wickerham's complete medium were unable to grow on Wickerham's medium when nitrous acid was employed as a mutagen, no colonies were detected which exhibited even a temporary sterol requirement. The use of ultraviolet irradiation as a mutagen, however, gave rise to several colonies exhibiting a sterol requirement as indicated by the replica plate screening technique. When the colonies from the master plates were streaked onto plates of Wickerham's complete agar without sterol supplements, only slight initial growth was observed. If a drop of an ethanolic solution was placed near the colonies, however, excellent growth was observed in two days. When these

colonies were transferred to WCY media supplemented with 5 μg/ml sterol, however, and replated onto Wickerham's complete agar with and without sterol supplements, equal numbers of colonies were observed on both types of media.

When sterol solubilized with the yeast extract complex was employed, numerous tentative sterol mutants were detected on the replica plates. Streaking of these clones onto Wickerham's complete agar with and without sterol supplements revealed excellent growth on the sterol supplemented medium and only poor initial growth on the nonsupplemented medium. However, many revertant, fast growing colonies were detected on the media without sterol and growth of the mutant strains in Wickerham's plus sterol broth or YCM broth revealed a complete reversion to wild type nonsterol requiring strains. Similarly, subculture on sterol supplemented YCC agar slants resulted also in a loss of the sterol requirement.

DISCUSSION

A component from yeast extract has been isolated which is capable of solubilizing exogenous ergosterol and cholesterol. This component was also found to be complexed with low levels of ergosterol as isolated from hot water extracts of whole yeast. The results of dialysis and the elution behaviour of the complex from Sephadex columns are evidence that the component is a macromolecule. The negative reaction of the complex to ninhydrin, the absence of Lowry protein color, the red coloration with iodine, the precipitation with alcohol and the positive reaction to the anthrone test for carbohydrates indicate the complex to be a polysaccharide. The results of acid hydrolysis and subsequent thin layer chromatography would indicate that the complex is composed solely of glucose subunits. As glycogen is the main carbohydrate reserve in yeast (21), it was thought that the complex may in fact be glycogen. However, the binding of ergosterol by glycogen appears to be neither as complete nor as consistent as the binding of sterol by yeast extract. Additionally, it can be noted from the data presented in Table 10 that the complexing agent is approximately only one-tenth as sensitive to the anthrone color reagent as glucose or glycogen. It should be noted here that the glycogen employed in these tests was prepared from oysters rather than from yeast,

commercial yeast glycogen being unavailable. Thus the possibility is not ruled out that the complexing agent is yeast glycogen or that it is a minor polysaccharide component, of which several are known in yeast (21). The exact nature of the complexing agent, however, will have to come from a thorough analysis of the linkages involved in the polysaccharide.

It is hoped that this water-soluble form of ergosterol will facilitate the investigation of the role of sterols in the physiology of yeast in both cell and cell-free systems. The advantage of using a natural component of the yeast system to solubilize sterol over the various emulsifiers currently employed is obvious. The most widely used of these emulsifiers is Tween-80. Festenstein (24) employed Tween-80 to suspend ergosterol for an investigation of the effect of ergosterol on anaerobic glycolysis in rat liver slices. In addition to the reported inhibition of glycolysis by the Tween-80-ergosterol suspension, he noted a substantial inhibition of glycolysis by the Tween-80 controls employed. This observation is not surprising considering the demonstration by Davis and co-workers (16) that oleic acid functions as an uncoupler of oxidative phosphorylation in rat liver mitochondria and that partial uncoupling resulted in a stimulation of gluconeogenesis. As previously mentioned, Tween-80 contains both free and esterified oleic acid. It can be appreciated that any attempt to study the effect of sterol on the

physiology of yeast using Tween-80 is subject to criticism because of the deleterious effects of the solubilizing agent on the metabolism of the organism.

As evidenced by the experiments with hexadecane and pulegone, yeast extract is evidently capable of binding nonsterolic compounds with some degree of specificity. The nature of this specificity, however, would require the testing of a much wider spectrum of compounds than those tested here and should include analysis by gel filtration.

From the data obtained by the six methods of sterol extraction it is obvious that the routine method of saponification is not recovering all of the sterol from the cells. The data suggest the existence of a form of sterol resistant to extraction following saponification but that is converted to an extractable form following treatment with acid.

The time course analyses of the sterol content in microaerobically cultured aerated suspensions exhibits the previously reported decline and subsequent increase of sterol content as determined by saponification and extraction. The data also reveal that the amount of sterol extracted from acid hydrolysed cells increased as the amount obtained from saponified cells decreased. When the sum of acid and base recoveries are plotted as shown in Figure 6, it can be seen that relatively smooth curves for sterol synthesis are

obtained from both the galactose and glucose grown cells.

The effect of the carbon source of the microaerobic cultures on the yield of sterol by the two methods shows that more acid labile sterol is obtained from the glucose grown cells than from the galactose grown cells. However, more base labile sterol is obtained from the galactose grown cells than from the glucose grown cells. Whether this phenomenon is due to some type of glucose effect which acts on the synthesis of enzymes concerned with the synthesis of base labile sterol but which exerts no such effect on the enzymatic mechanisms responsible for the acid labile form is not known.

The decline in base labile sterol coinciding with the exhaustion of the fermentable carbon source from the medium occurs during the shift from a fermentive to a respiratory metabolism (64). As a consequence, it would be expected that a substrate metabolized only by respiration should not show a loss in base extractable sterol. This is supported by the observation that acetate, when substituted for glucose in the aeration medium, shows no such decline in the base labile sterol content of the cells.

These data give strong presumptive evidence for dual forms of ergosterol; one which is base labile, the other which is acid labile. Interconvertibility of the two forms is suggested. If one is to propose a role for such an acid labile form of sterol in the metabolism of the cell, one must consider the observations that

this acid labile form is predominant in the early stages of adaptation to aerobic respiration and that this form increases as the base labile form decreases upon the exhaustion of glucose from the aeration medium.

The experiments dealing with the effect of respiratory inhibitors on the time course sterol content of aerated suspensions suggest a number of interesting hypotheses concerning the relationship between sterol synthesis and metabolism and the respiratory state of the cell. The fact that chloramphenicol has such a marked inhibitory effect on sterol synthesis indicates that, despite the fact that no increase in cell numbers is observed in the course of the aeration, protein synthesis on the 70S ribosomal system of the mitochondrion is being carried out, the required amino acids perhaps being obtained by a scavenging of other cellular components. This inhibition would also suggest that at least some of the enzymes involved in sterol synthesis are synthesized on the 70S ribosomal system. This is not surprising considering that many of the enzymes involved in the conversion of lanosterol to ergosterol are thought to be of a particulate nature. The possibility that the effect of chloramphenicol on sterol synthesis is a secondary one caused primarily by the inhibition of the respiratory apparatus by the drug would seem to be ruled out by the fact that respiratory deficient petite strains synthesize sterol in amounts equivalent to wild type

respiratory competent strains. A point to be considered, however, is the possibility that the chloramphenicol in such high concentrations is exerting some kind of an inhibitory effect on sterol synthesis which is distinct from its action as an inhibitor of protein synthesis.

The addition of cold methionine to aerating cell suspensions after eight hours of aeration produces a slight decrease in the labeled sterol content of the cells. Whether this is a reflection of a small amount of degradation of ergosterol or whether it is due to an induced conversion of the sterol to a form resistant to saponification is unknown.

The experiment dealing with the effect of Metrazol on the time course analysis of sterol content reveals a much less severe inhibition of sterol synthesis than that observed by Alexander and coworkers (3). There is no apparent effect on sterol content of the cells aerated in the presence of the drug and the effect on cells grown in the presence of the drug appears limited to a slight reduction in total sterol content and in an elimination of the decline and subsequent increase observed in the control cells. Evidently the drug must be incorporated into some subcellular unit to exert its inhibitory effect.

The effect of acriflavine on the sterol content of cells presented in Figure 10 presents a similar picture; there is no apparent effect

on the cells aerated in the presence of the inhibitor, and there is a slight reduction in the sterol content of the cells grown in the presence of acriflavine. However, if one examines the data presented in Figure 12 with cultures employing larger cell concentrations with the control cells exhibiting the decline and subsequent increase in sterol content, it can be seen that whereas the control cells show a decline at 16 hours, the cells aerated with acriflavine continue to increase in sterol content at 12 hours after which the content gradually declines. This phenomenon is explainable if one considers the evidence (58) that the β-fraction of mitochondrial DNA is encoded for the enzymes necessary for respiratory competency and that yeast aerated in the presence of acriflavine do not become respiratory competent (60). If the hypothesis that the adaptation from a fermentive metabolism to a respiratory metabolism is intimately related to the decline and subsequent increase in base labile sterol is correct, the "overshoot" of base labile sterol detected in the presence of the acriflavine can be explained by the hypothesis that the conversion from the base labile to an acid labile form does not occur because the adaptation to a respiratory competent state does not occur in the presence of acriflavine. The observed lower sterol content of cells grown in the presence of the inhibitor may reflect the lower respiratory state of these cells. It should be remembered that these cells have probably been

entirely converted into petites because of growth in the presence of the mutagen. The lower sterol content of cells grown in acriflavin or in Metrazol, both potent respiratory inhibitors, suggests that the amount and perhaps the form of the sterol synthesized is at least in part dependent upon the development of the respiratory apparatus.

Parks and Starr (64) observed a 12.5% increase in the sterol content of cells aerated in the presence of sodium azide. The data presented in Figure 9 demonstrate an almost two-fold increase in the sterol content of cells grown in the presence or absence of azide and aerated in its presence. It will again be noticed that the cells grown in the presence and aerated in the absence of the inhibitor have a slightly lower sterol content than the control cells. This may again reflect the lower respiratory state of the cells. The effect of the azide included in the medium on cells being aerated is quite remarkable. The observed increase in sterol content could be due either to an increased synthesis of sterol or it could be due to the release of sterol previously present in an alkali resistant form by the presence of the sodium azide. It will be recalled that a 62% increase in extractable sterol was obtained from cells which had been exposed to dilute HCl prior to saponification. Sodium azide is known to be an uncoupler of oxidative phosphorylation. Unlike dinitrophenol, which is thought to inhibit

oxidative phosphorylation by interaction with or cleavage of high energy intermediates leading to the phosphorylation of ADP to ATP, sodium azide is believed to act as an uncoupler by effecting small changes in the structural relationships between high energy intermediates and the soluble factors involved in oxidative phosphorylation. The effect of the azide is seen as disrupting the structural integrity of the phosphorylating particle, causing a small structural displacement and thus uncoupling the process of oxidative phosphorylation (67, p. 146-151).

Considering this postulated mode of action of azide on the structural integrity of the phosphorylating particles and the observed additional release of sterol upon treatment with acid, it is tempting to hypothesize that increased extractable sterol content of cells observed in the presence of sodium azide is due to the release of sterol from the lipoprotein particles. Although lipoprotein particulate fractions containing ergosterol have been isolated from yeast (61), the possible structural or enzymatic role of sterols in the mechanism of electron transport or oxidative phosphorylation has apparently been given little consideration. In a review of the components of the respiratory chain, Racker (67, p. 93-107) does not mention the presence of sterols in mitochondria. If azide does in fact act to release additional sterol upon saponification, exposure of previously aerated cells to azide in the absence of oxygen should

show an increase in extractable sterol following saponification, when compared to control cells not exposed to the presence of azide.

That sterol is present in mitochondria and may in fact play some role in the respiration of yeast would not be unexpected. The fact that sterol can account for up to 10% of the dry weight of cells under maximal conditions suggests a possible structural role for the sterol. The sparing effect of ergosterol on the induction of petite mutants in cultures grown at high temperatures may be due to the requirement for the sterol, which cannot be synthesized at high temperatures. Ergosterol would be required for the maintenance of a structural continuity similar to that observed by pantothenate deficient cultures. Additionally, the demonstration by Lukins and co-workers (47) that ergosterol included in the medium of microaerobically grown glucose derepressed yeast results in a profound change in succinic dehydrogenase activity and the cytological characteristics of the cells suggests a role for the sterol in the maintenance of respiratory competency. Similarly Morpurgo and co-workers (56) observed the development of typical mitochondrial structures, when ergosterol was included in the microaerobic growth medium. A correlation also exists in the observation by Polakis and co-workers (65) of the development of maximal respiratory activity in the late stages of the growth cycle and the fact that yeast attain a maximal capacity for sterol synthesis in the stationary phase of growth (49).

Moustacchi and Williamson (58) have shown that two mitochondrial DNA fractions, γ and β, decrease in amount relative to the total cellular DNA in the early stages of growth where glucose repression is maximal and respiratory activity minimal and subsequently increase as the cells enter the stationary phase of growth. The β fraction has been associated with the synthesis and control of the respiratory apparatus. The authors postulate no role for the γ fraction. However, the evidence that ergosterol plays a role in the mitochondrion, that the enzymes involved in the synthesis and control of sterol metabolism are synthesized on the 70S ribosomal system of the mitochondrion and that the γ fraction varies in a similar manner to the β fraction suggests the possibility that the observed γ fraction encodes for the enzymes concerned with the synthesis and or regulation of sterols in yeast.

That the γ fraction may in fact play a role in sterol metabolism in yeast is supported by considering what has been found during attempts to isolate a sterol mutant in yeast. Extensive efforts to isolate a sterol mutant have resulted only in the isolation of a few clones exhibiting a temporary requirement for sterol. If one assumes that the genetic information for sterol synthesis is carried on the γ DNA fraction of the mitochondrion, the difficulty of obtaining even temporary sterol mutants is easily understood. Yeast cells contain several mitochondria and thus it would be necessary to

induce mutations in all of the copies of the γ DNA present to effect a sterol requirement in any given cell. The reversion of the temporary mutants upon subculture could be explained in light of the recent demonstration by Thomas and Wilkie (95) of mitochondrial recombination of drug-resistance factors in yeast. They conclude that nonchromosomal recombination of diploid sensitive and resistant strains is due to crossing over between strands of mitochondrial DNA. Such recombination between mutant mitochondrial DNA strands within a yeast cell auxtotrophic for sterol or with a prototrophic cell could result in the observed reversion to nonsterol requiring clones.

The exact role of ergosterol in the metabolism of yeast is still unknown. The hypothesis that the sterol is intimately involved in, and required for, respiration is strengthened by the evidence for an acid labile form of the sterol. Whether this form is involved in the structure and function of the respiratory chain remains to be demonstrated. The hypothesis that the γ fraction of mitochondrial DNA is involved in sterol metabolism would seem to warrant further investigation. It is hoped that this hypothesis might provide the basis for a new approach to the problem of isolating a sterol mutant. The role of sterols in the yeast cell membrane and the existence of a steryl glucoside remains an area of future investigation. Finally, the role of the soluble ergosterol complex in the metabolism of the cell remains undefined.

SUMMARY

A water-soluble complex containing ergosterol together with a component of yeast has been isolated. The complex can be isolated from commercial yeast extract to which ergosterol has been added or directly from whole yeast cells. The complexing agent from yeast extract is also capable of solubilizing cholesterol and a long chain hydrocarbon, hexadecane. The complexing agent has been shown to be a polysaccharide and appears to be composed solely of glucose subunits. The complexing agent does not appear to be glycogen. The binding between the sterol and the polysaccharide appears to be noncovalent. The complex is easily prepared and is stable in aqueous solution; ergosterol in this solution is metabolically available to yeast cells to which it is added.

Data obtained from acid hydrolysis and extraction of yeast have demonstrated that routine saponification does not recover total sterol from the cells. This suggests the existence of a form of ergosterol resistant to saponification. Time course analyses of sterol synthesis by nonproliferating cell suspensions reveal an inverse relationship between the amounts of base labile and acid labile forms of sterol. These data give strong presumptive evidence for dual forms of ergosterol which are interconvertible according to the respiratory state of the cell.

Experiments dealing with the effect of respiratory inhibitors on sterol synthesis in nonproliferating cell suspensions suggest that the synthesis and physiological form of ergosterol is intimately related to the integrity of the respiratory apparatus and that the DNA encoding for the synthesis and regulation of ergosterol is located in the mitochondria.

BIBLIOGRAPHY

1. Aaronson, S. A role for a sterol and a sterol precursor in the bacterium Rhodopseudomonas palustris. Journal of General Microbiology 37:225-229. 1964.

2. Adelberg, E. A., et al. Attempted induction of microbial mutants requiring steroidal growth factors. Journal of Bacteriology 69:733. 1955.

3. Alexander, R. B., F. C. Brand and G. J. Alexander. Effect of pentamethlenetetrazol on structure and enzyme activity of yeast. I. Changes in mitochondria and in sterol levels. Biochemica et Biophysica Acta 111:318-325. 1965.

4. Andreason, A. A. and T. J. B. Stier. Anaerobic nutrition of Saccharomyces cerevisiae. I. Ergosterol requirement for growth in a defined medium. Journal of Cellular and Comparative Physiology 41:23-26. 1953.

5. Andreason, A. A., and T. J. B. Stier. Anaerobic nutrition of Saccharomyces cerevisiae. II. Unsaturated fatty acid requirement for growth in a defined medium. Journal of Cellular and Comparative Physiology 43:271-282. 1954.

6. Aronoff, S. Techniques of radiobiochemistry. Ames, Iowa State College Press, 1956. 228 p.

7. Axelrod, L. R. The quantitative separation of estrogens by paper partition chromatography. Journal of Biological Chemistry 201:59-69. 1953.

8. Bechet, J. and J. M. Wiame. Indication of a specific regulatory binding protein for ornithinetranscarbamylase in Saccharomyces cerevisiae. Biochemical and Biophysical Research Communications 21:226-234. 1966.

9. Cedillos, R. A., R. E. Reeves and J. C. Swartzwelder. Influence of added cholesterol and dihydrocholesterol upon multiplication of Entamoeba Histolytica in MS-F medium. Experimental Parasitology 11:305-310. 1961.

10. Chattaway, F. W., J. D. Townsley and A. J. E. Barlow. The effect of certain steroids on the growth of Trichophyton rubrum. Journal of General Microbiology 28:437-441. 1962.

11. Corwin, L. M., L. J. Schroeder and W. G. McCullough. Studies of lipide synthesis in cell-free yeast extracts. Archives of Biochemistry and Biophysics 72:112-118. 1957.

12. Clark-Walker, G. D. and A. Linnane. The biogenisis of of mitochondria in Saccharomyces cerevisiae. A comparison between cytoplasmic respiratory-deficient mutant yeast and chloramphenicol inhibited wild type cells. Journal of Cell biology 34:1-14. 1967.

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

14. Cornforth, J. W. Biosynthesis of fatty acids and cholestrol considered as a chemical process. Journal of Lipid Research 1:3-28. 1959.

15. Danielson, H. and K. Bloch. On the origin of the C-28 in ergosterol. Journal of the American Chemical Society 79: 500-501. 1957.

16. Davis, E. J. and D. M. Gibson. Stimulation of phosphoenol-pyruvate formation by oleic acid and other uncouplers of oxidative phosphorylation in rabbit liver mitochondria. Biochemical and Biophysical Research Communications 29: 815-821. 1967.

17. Deborin, G. A. and L. B. Gorbacheva. Protein-lipide complexes and their properties. Biokhimiya 18:618-625. 1953. (Abstracted in Chemical Abstracts 48:4667g. 1954)

18. Deborin, G. A. and O. M. Shibanova. Complexes of protein with lipids and their properties. Viscosity of egg albumin and its complex with ergosterol. Doklady Akademii nauk S S R 98:241-242. 1954. (Abstracted in Chemical Abstracts 49: 1836i. 1955)

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

20. Dulaney, E. L., E. O. Staply and K. Simpf. Ergosterol production by yeasts. Applied Microbiology 2:371-379. 1954.

21. Edwards. T. E. Yeast polysaccharides. Advances in Carbohydrate Chemistry 5:176-179. 1965.

22. Ephrussi, B. Nucleo-cytoplasmic relationships in microorganisms: their bearing on cell heredity and differentiation. Oxford, Clarendon, 1953. 127 p.

23. Ephrussi, B. and H. Hottinguer. On an unstable cell state in yeast. Cold Spring Harbor Symposia on Quantitative Biology 16:75-84. 1951.

24. Festenstein, G. N. Effect of ergosterol and ergocalciferol on the anaerobic glycolysis of rat liver slices. Biochemical Journal 59:605-609. 1955.

25. Fiertel, A. and H. P. Klein. On sterols in bacteria. Journal of Bacteriology 78:738-739. 1959.

26. Fieser, L. G. and M. Fieser. Steroids. New York, Reinhold, 1959. 945 p.

27. Gal'tsova, R. D., A. T. Novichkova and I. P. Vakina. Effects of glucose on ergosterol synthesis by yeast. Mikrobiologiya 28:502-506. 1959. (Abstracted in Chemical Abstracts 53:22252h. 1959)

28. Gawel, L. J., J. R. Turner and L. W. Parks. Accumulation of S-adenosylmethionine by microorganisms. Journal of Bacteriology 83:497-499. 1962.

29. Gerard, E. Sur les cholesterine des crytogames. Journal de Pharmacie et de Chimie 1:601-608. 1895.

30. Halevry, S. and O. Gisry. Lipid composition of Trypanosoma ranarum. Poceedings of the Society for Experimental Biology and Medicine 117:552-55. 1964.

31. Halevry, S. and S. Sarel. Isolation of ergosterol from the trypanosamatid Leptomonas culicidarum. Journal of Protozoology 12:293-296. 1965.

32. Heftman, E. Chromatography. New York, Reinhold, 1967. 851 p.

33. Issidarides, C. H., I. Kitigawa and E. Mosettig. Cleavage of

steroidal digitonides in dimethyl sulfoxide. Journal of Organic Chemistry 27:4693-4694. 1962.

34. Johnston, J. A. Effects of streptomycin on the biosynthesis of ergosterol in growing cultures and cell-free preparations of yeast. Antimicrobial Agents Chemotherapy 1961:254-259. 1962.

35. Klein, H. P. Synthesis of lipid in resting cells of Saccharomyces cerevisiae. Journal of Bacteriology 69:620. 1955.

36. Klein, H. P. and Z. K. Booher. Synthesis of lipids in cell-free extracts of yeast. Proceedings of the Society for Experimental Biology and Medicine 89:43-44. 1955.

37. Klein, H. P., N. R. Eaton and J. C. Murphy. Net synthesis of sterols in resting cells of Saccharomyces cerevisiae. Biochimica et Biophysica Acta 13:591. 1954.

38. Klein, H. P. and S. Greenfield. Effects of mitochondria on lipid synthesis in yeast homogenates. Experimental Cell Research 17:185-188. 1959.

39. Klein, H. P. and F. Lipman. The relationship of Co A to lipid synthesis. Journal of Biological Chemistry 203:95. 1953.

40. Kodicek, E. Biosynthesis of yeast sterols and preparation of C^{14}- labelled vitamin D_2. In: CIBA Foundation Symposium on the Biosynthesis of Terpenes and Sterols, ed. by G. E. W. Wolstensholme and C. M. O'Connor. Boston, Little Brown, 1958. p. 173-183.

41. Kovac, L. et al. On the relationship between respiratory activity and lipid composition of the yeast cell. Biochimica et Biophysica Acta 144:94-101. 1967.

42. Lampen, J. D. et al. Location and role of sterol at nystatin-binding sites. Journal of Bacteriology 84:1152-1160. 1962.

43. Lederberg, J. and E. M. Lederberg. Replica plating and indirect selection of bacterial mutants. Journal of Bacteriology 63:399-406. 1952.

44. Lerman, L. S. Structural considerations in the interaction of DNA and acridines. Journal of Molecular Biology 3:18-30. 1961.

45. Loginova, L. G., N. F. Gerasimova and L. M. Seregina. Supplementary growth factor requirements of thermotolerant yeasts. Mikrobiologiya 31:29-34. 1962. (Abstracted in Chemical Abstracts 57:5111g. 1963)

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

47. Lukins, H. B. et al. Correlation of membrane bound succinate dehydrogenase with the occurrence of mitochondrial profiles in Saccharomyces cerevisiae. Biochemical and Biophysical Research Communications 23:363-367. 1967.

48. Mackler, B. et al. Biochemical correlates of respiratory deficiency. IV. Composition and properties of respiratory particles from mutant yeasts. Biochemistry 4:2016-2020. 1965.

49. Madhyastha, P. Postdoctoral Fellow, Oregon State University, Dept. of Microbiology. Personal communication. Corvallis, Oregon. February 7, 1967.

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

51. Massengale, O. N., C. E. Bills and P. S. Prickett. Factors determining the ergosterol content of yeast. II. Carbohydrate sources. Journal of Biological Chemistry 94:213-219. 1931.

52. Matkovics, B. Action of sterols on the growth of microorganisms. Pharmazie 15:122-125. 1960. (Abstracted in Chemical Abstracts 54:17455d. 1960)

53. Matkovics, B. and A. Konschansky. The action of the steroids on microorganisms. II. The action of ergosterol on the oxidation-reduction potential and dry weight of Penicillium chrysogenum, strain Q 176. Zentralblatt für Bakteriologie, Parasitenkunde, infektionskrankheiten und Hygiene 111:543-545. 1958. 25058i. 1960)

54. Matkovics, B. and G. Sipos. Effect of sterols on the growth of microorganisms. Acta Universitatis szegediensis, Acta biologica 5:25-30. 1959. (Abstracted in Chemical Abstracts 54:25058i. 1960)

55. Miyazaki, T., Y. Amano and S. Miinobe. Isolation of ergosterol palmitate from Penicillium chrysogenum Q-176. Tokyo Yakka Daigku Nempo 12:54-55. 1962. (Abstracted in Chemical Abstracts 59:9277f. 1963)

56. Morpurgo, G. et al. The influence of ergosterol on the physiology and the ultrastructure of Saccharomyces cerevisiae. Nature 201:897-899. 1964.

57. Mounolou, J. C., H. Jakob and P. P. Slonimski. Mitochondrial DNA from yeast "petite" mutants: specific changes of buoyant density corresponding to different cytoplasmic mutations. Biochemical and Biophysical Research Communications 24:218-224. 1966.

58. Moustacchi, E. and D. H. Williamson. Physiological variations in sattelite components of yeast DNA detected by density gradient centrifugation. Biochemical and Biophysical Research Communications 23:56-61. 1966.

59. Nagai, S. Induction of the respiration deficient mutation in yeast by various synthetic dyes. Science 130:1188-1189. 1959.

60. Nagai, S., N. Yanagishima and H. Nagai. Advances in the study of respiration-deficient (RD) mutation in yeast and other microorganisms. Bacteriological Reviews 25:404-426. 1961.

61. Nyman, M. A. and E. Chargaff. On the lipoprotein particles of yeast cells. Journal of Biological Chemistry 180:741-746. 1949.

62. Oparin, A. I., N. S. Gelman and G. A. Deborin. The significance of structure in certain enzymic processes. Archives of Biochemistry and Biophysics 69:582-591. 1957.

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

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

65. Polakis, E. S., W. Bartley and G. A. Meek. Changes in the structure and enzyme activity of Saccharomyces cerevisiae in response to changes in the environment. Biochemical Journal 90:369-374. 1964.

66. Raab, W. Antibacterial action of phenanthrene related substances. Science 103:670-671. 1946.

67. Racker, E. Mechanisms in bioenergetics. New York, Academic, 1965. 259 p.

68. Rajagopalan, K. V. and P. S. Sarma. Effects of sulfanilamide on formation of sterols in Saccharomyces cerevisiae and Neurospora crassa. Biochemical Journal 69:53-56. 1958.

69. Resnick, M. A. and R. K. Mortimer. Unsaturated fatty acid mutants of Saccharomyces cerevisiae. Journal of Bacteriology 92:597-600. 1966.

70. Rothblat, G. H. and P. F. Smith. Nonsaponifiable lipids of representative pleuropneumonia-like organisms. Journal of Bacteriology 82:479-491. 1961.

71. Ruyssen, R. and J. Maebe. Solubilization of cholesterol in solutions of sodium dodecyl sulfate and sodium deoxycholate. Mededelingen van de K. Vlaamscha academie voor wetenschapen 15:3-16. 1953. (Abstracted in Chemical Abstracts 48:5265f. 1954)

72. Sarachek, A. and G. L. Fowler. Induction of heritable respiratory deficiency in Saccharomyces by pantothenate starvation. Nature 190:792-794. 1961.

73. Sherman, F. The effects of elevated temperature on Yeast I. Nutrient requirements for growth at elevated temperatures. Journal of Cellular and Comparative Physiology 54:29-35. 1959.

74. Sherman, F. The effects of elevated temperatures on yeast II. Induction of respiratory-deficient mutants. Journal of Cellular and Comparative Physiology 54:37-52. 1959.

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

76. Smith, P. F. Cholesterol esterase activity of pleuropneumonia-like organisms. Journal of Bacteriology 77:682-689. 1959.

77. Smith, P. F. Fate of ergosterol in pleuropneumonia-like organisms. Journal of Bacteriology 84:534-538. 1962.

78. Smith, P. F. The role of sterols in the growth and physiology of pleuropneumonia-like organisms. In: Recent progress in microbiology: Symposia held at the Eighth International Congress for Microbiology, Montreal, 1962. Springfield, Illinois, Thomas, 1963. p. 518-525.

79. Smith, P. R. The carotenoid pigments of Mycoplasma. Journal of General Microbiology 32:307-319. 1963.

80. Smith, P. F. and R. J. Lynn. Lipid requirements for the growth of pleuropneumonia-like organisms. Journal of Bacteriology 76:264-269. 1958.

81. Smith, P. F. and G. H. Rothblat. Incorporation of cholesterol by pleuropneumonia-like organisms. Journal of Bacteriology 80:842-850. 1960.

82. Smith, P. F. and G. H. Rothblat. Comparison of lipid composition of pleuropneumonia-like and L-type organisms. Journal of Bacteriology 83:500-506. 1962.

83. Somlo, M. and H. Fukuhara. On the necessity of molecular oxygen for the synthesis of respiratory enzymes in yeast. Biochemical and Biophysical Research Communications 19: 587-591. 1965.

84. Starr, P. R. and L. W. Parks. Effect of temperature on sterol metabolism in yeast. Journal of Cellular and Comparative Physiology 59:107-110. 1962.

85. Starr, P. R. and L. W. Parks. Some factors affecting sterol formation in Saccharomyces cerevisiae. Journal of Bacteriology 83:1042-1046. 1962.

86. Stern, A. I., J. A. Schiff and H. P. Klein. Isolation of ergosterol from Euglena gracilis. Distribution among mutant strains. Journal of Protozoology 7:52-55. 1960.

87. Stewart, J. M. and D. W. Woolley. Antimetabolites of melvalonic acid. II. Inhibition of ergosterol synthesis in yeast. Biochemistry 3:1998-2004. 1964.

88. Swell, L. and C. R. Treadwell. Cholestrol esterases VI. Relative specificity and activity of pancreatic cholesterol esterases. Journal of Biological Chemistry 212:141-150. 1955.

89. Talalay, P. and H. G. Williams-Ashman. Activation of hydrogen transfer between pyridine nucleotides by steroid hormones. Proceedings of the National Academy of Sciences 44:15-26. 1958.

90. Tanret, C. Sur un nouveau principe immediat des l'ergot de sigle, l'ergosterine, Comptes Rendus Hebdomadaires des Siances de L'Academie des Sciences 108:98. 1889.

91. Tauson, T. A. Distribution and conditions for accumulation of ergosterol (provitamin-D_2) in yeast organisms. I. Content of ergosterol in yeast organisms. Mikrobiologiya 11: 46-58. 1942. (Abstracted in Chemical Abstracts 38:3312. 1944)

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

93. Tchenn, T. A. and K. Bloch. Role of oxygen and reduced pyridine nucleotides in the biogenisis of cholesterol. Abstracts of Papers of the American Chemical Society 130: 56c-57c. 1956.

94. Tewari, K. K., J. Jayaraman and H. R. Mahler. Separation and characterization of mitochondrial DNA from yeast. Biochemical and Biophysical Research Communications 21: 141-148. 1965.

95. Thomas, D. Y. and D. Wilkie. Recombination of mitochondrial drug-resistance factors in Saccharomyces cerevisiae. Biochemical and Biophysical Research Communications 30: 368-372. 1968.

96. Trufanov, A. V. and V. A. Kirsanova. Ergosterol content of yeast during autolysis. Biokhimiya 4:377-380. 1939. (Abstracted in Chemical Abstracts 34:1708-6. 1940)

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

98. Tustanoff, E. R. and W. Bartley. The effect of glucose on the development of respiration by anaerobically grown yeast. Canadian Journal of Biochemistry and Physiology 42:651-665. 1964.

99. Vendt. V. P. Protein-sterol complexes. Vitaminy, Adademiya Nauk Ukrainskoi S S R, Sbornik 2:30-39. 1956. (Abstracted in Chemical Abstracts 51:18037f. 1957)

100. Wickerham, J. A. A critical evaluation of the nitrogen assimilation tests commonly used in classification of yeasts. Journal of Bacteriology 52:293-301. 1946.

101. Williams, B. L., T. W. Goodwin and J. F. Ryley. Sterol content of some protozoa. Journal of Protozoology 13:227-230. 1966.

102. Windaus, A. and O. Rygh. Esters of ergosterol and their behaviour toward ultraviolet irradiation. Nachrichten von der gesellschaft der Wissenschaften zu Göttingen. Geschäftliche mitteilungen; Mathematisch-Physikalische Klasse 2:202-216. 1928. (Abstracted in Chemical Abstracts 24:4789. 1930)

103. Ycas, M. A hereditary cytochrome deficiency appearing in yeast grown at an elevated temperature, Experimental Cell Research 10:746. 1956.