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Title: A ROLE FOR STEROLS AS MODULATORS OF MEMBRANE FLUIDITY

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The role of sterols as structural components in the mitochondrial membrane of Saccharomyces cerevisiae was investigated. Gel to liquid-crystalline transitions of mitochondrial membrane preparations from mutant and wild type cells were probed using the technique of fluorescence polarization. Mitochondria isolated from those strains accumulating sterols other than ergosterol exhibited a phase transition; wild types did not. The technique was extended to an artificial membrane system using a series of sterols with modifications in the hydroxyl moiety, the ring system, and the side chain to investigate the contribution of various sterol structures to membrane fluidity. Sterols possessing a planar ring, a β-hydroxyl and a saturated side chain conferred maximal order to the bilayer. Arrhenius kinetics of the mitochondrial membrane-bound enzyme L-kynurenine-3-hydroxylase paralleled changes in fluidity observed in the fluorescence study. Examination of sphaeroplast and mitochondrial response to osmotic strength showed no correlation between sterol structure and ability to resist stretching. Uptake studies of the lipophilic cation methyltriphenylphosphonium by the mitochondria enabled the calculation of the transmembrane potential. Potentials for mitochondria isolated from organisms

accumulating sterols with side chain modifications were ten millivolts lower than those from the wild type strains. No variation of respiratory competency as a function of sterol composition was discerned. The observations are discussed in relation to an adjustment of the membrane properties to accomodate sterols other than ergosterol without extensive alteration of enzymatic activities and permeability properties. The organisms used were characterized as to sterol to phospholipid ratio and fatty acid composition.

A Role for Sterols as Modulators of Membrane Fluidity

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I. Introduction

A great deal of effort has been expended to elucidate the cellular role of sterols. The hypothesis that they function solely as precursors of the steroid hormones has been replaced by the supposition that sterols serve as structural components of membranes. Similiarly, the concept of the membrane simply as an inert barrier separating the cell from its surroundings has been superseded by a model in which the membrane has a dynamic role affecting a variety of cellular processes. The composition of the membrane lipid bilayer alters the activity of membrane bound enzymes as well as the spatial orientation of intrinsic multi-enzyme complexes. The passive permeability of the bilayer to a variety of solutes is also dependent on the constituents of the membrane. On the microscopic scale the bilayer may physically be in a gel or liquid-crystalline phase, depending on the temperature of the system as well as the composition of the bilayer. These many functions and properties of the membrane provide a variety of approaches for the study of the effects of sterol on the bilayer at the physical, biochemical, and functional levels. A plethora of studies on the effect of sterol additional to artificial bilayers has been reported, but few attempts have been made to clarify the results of qualitative sterol alteration on the overall cellular physiology. This thesis attempts such an investigation.

II. Survey of the Literature

The mitochondrial membrane of Saccharomyces cerevisiae provides an invaluable system for the examination of the role of sterols in membranes. The organism itself is easily cultivated and has yielded a variety of sterol mutant strains. Being a facultative anaerobe, it does not require oxidative metabolism. This feature enables the manipulation of the organism and culture conditions to produce extreme alterations in mitochondrial function for experimentation.

The mitochondrial membrane of this organism is of particular interest because it exists in two well-defined physiologic states. Under aerobic conditions, the organelle appears as a double membraned structure on electron microscopic examination. The inner membrane is highly invaginated, forming well developed cristae. Associated with this structure is a cyanide sensitive respiratory chain which includes cytochromes a, a_3, b, c_1, and c as well as a host of enzymatic activities (cytochrome c oxidase, NADH-oxidase, succinate oxidase, succinate-cytochrome c reductase, succinate-ferricyanide reductase, NADH-ferricyanide reductase, succinate dehydrogenase, F_1-ATPase, L-kynurenine-3-hydroxylase, sterol methyltransferase, etc.). Under anaerobiosis, the structure formed is less differentiated and is known as a promitochondrion. The organelle retains its double membraned characteristic, but lacks the well developed cristae characteristic of the aerobically produced mitochondria. Also absent are many of the enzymatic activities as well as the respiratory chain and many of the cytochromes. The structure retains the F_1-ATPase,

NADH-ferricyanide reductase, sterol methyltransferase, and succinate dehydrogenase activities as well as mitochondrial DNA. Criddle and Schatz[1] have shown the two structures to be analogous.

The lipid composition of mitochondrial and promitochondrial membranes have been examined[2,3,4]. The fatty acid composition differs in the two states. Unsaturated, short chained fatty acids, generally less than 14 carbons in length, are the predominant species in the promitochondrion, whereas oleic and palmitoleic are the dominant species in the mitochondria. The phospholipids are also altered, there being a greater proportion of phosphatidyl inositol and a lesser proportion of phosphatidyl ethanolamine in the anaerobic condition. Whereas the amount of neutral lipid remains constant in both states, the amount of ergosterol decreases from 30 micrograms per milligram of protein in the aerobic condition to less than five micrograms per milligram of protein in the anaerobic condition. In making the transition from anaerobic to aerobic growth the cell expends a great deal of energy to alter its lipid composition. The production of sterol alone represents a large investment of energy by the cell and suggests a dynamic role for sterol in mitochondrial membrane development.

Saccharomyces cerevisiae has been shown by several investigators to have a sterol requirement for growth, of which the major sterol is ergosterol. Two steps in the sterol biosynthetic pathway, the cyclization of squalene to squalene-2,3-epoxide and the demethylation of lanosterol, require molecular oxygen.[5] Under anaerobic

conditions, no sterol is synthesized[6], and the organism is dependent on the addition of sterol to the medium.

Brockmann and Stier[7] observed a diminished cell yield following anaerobic growth as compared to aerobic growth. Using a defined medium without sterol supplement, Andreasen and Stier[8] showed the anaerobic culture produced only one or two generations, reaching a final concentration of 1-2 x 10^6 cells per milliliter of culture as contrasted to 400 x 10^6 cells per milliliter aerobically. Ergosterol supplementation of anaerobic cultures partially alleviated the restrictive growth condition and raised the cell yield to 110-125 x 10^6 cells per milliliter of culture. The history of the inoculum used for anaerobic growth was observed to affect the number of cells produced, an anaerobic inoculum producing a smaller cell yield than an aerobic culture.

Morpurgo et al.[9] confirmed this observation and reported a dependence of the final anaerobic cell yield on the concentration of cells in the inoculum, the higher the cell concentration in the inoculum the higher the cell yield. Furthermore, the number of viable cells in the anaerobic culture was inversely proportional to the number of cell divisions. Cell death resulted from lysis, suggesting that a defective membrane was produced under extended anaerobiosis. These observations indicate a dilution of sterol under conditions of anaerobiosis until the amount of ergosterol per cell reaches a critically low level, at which time growth stops. As the sterol to cell ratio decreases, the cells become more

susceptible to lysis. An anaerobic inoculum already has a diminished sterol to cell ratio which limits the number of divisions that the inoculum can support and produces a population with a shortened lifetime.

Accumulation of a large body of evidence indicates that catalytic and Arrhenius kinetics of intrinsic enzymes are affected by membrane composition[10,11,12,13,14,15]. Some enzymes appear to have more stringent environmental requirements than others. The modification of lipid composition as well as increased diversification of enzymatic activities during adaptation to aerobic growth suggests a dependence of the new enzyme activities on the lipid composition. Presumably, the altered membrane lipids optimize enzyme activities and spatial orientation within multienzyme complexes. If changes in temperature do not inactivate an enzyme, alter its affinity for substrate, or alter its optimal pH requirement, then the relationship between the rate of the reaction and the temperature may be determined from the Arrhenius equation, which can be expressed as the activation energy. The activation energy remains constant in many cases, and in plotting the log of the rate versus the reciprocal of the absolute temperature, a straight line results. However, in examining membrane associated functions, a discontinuity often occurs, indicating a change in activation energy. Studies indicate this change is the result of a transformation in the phase of the membrane from an ordered crystalline-like gel state to a disordered fluid state at higher temperatures[16,17]. Presumably the phase change produces a modification in the configuration of the enzyme, thereby altering the activation energy.

Ainsworth et al.[18] established the sensitivity of cytochrome c oxidase, an enzyme located on the inner mitochondrial membrane, to its lipid environment using a fatty acid desaturase mutant. Synthesis of unsaturated fatty acids is prevented by the genetic lesion and the mutant is dependent on an exogenous supplementation of unsaturated fatty acids. An Arrhenius plot of enzyme activity exhibited a discontinuity which was altered by the species of fatty acid supplemented during growth of the organism. Supplementation with oleic acid produced a discontinuity at 10.2°C whereas linoleic reduced the transition to 7.4°C.

Thompson and Parks[19] showed the activation energy for cytochrome c oxidase was also sensitive to alteration in sterol composition. A wild type strain in which ergosterol was the predominant sterol showed a transition temperature of 10.0°C which was in agreement with that reported by Ainsworth et al.[18]. The mutant strains, nys-3 and 3701b-n3, accumulate ergosta-7,22-diene-3β-ol instead of ergosterol. Cytochrome c oxidase activity in these mutants exhibited transitions at 4°C. Lipid analysis showed similar sterol levels and fatty acid compositions in the three organisms. The S-adenosylmethionine: Δ^{24}-sterol methyltransferase, also associated with the inner mitochondrial membrane, was examined. The enzyme isolated from the wild type showed a transition at 8.5°C whereas that isolated from the mutants exhibited a transition at 5°C.

Cobon and Haslam[20] manipulated the amount of sterol by growing Saccharomyces cerevisiae anaerobically with excess supplements of

unsaturated fatty acids and a range of ergosterol supplements. Ergosterol levels varied from 0.53 to 3.25 milligrams per gram dry weight for the whole cells and from 7.3 to 10.5 milligrams per gram of protein for the mitochondria. The activity of F_1-ATPase was similar over the range of sterol levels. However, Arrhenius plots showed a decrease in the temperature of the phase transition as the amount of sterol in the mitochondria was increased. A reduction in membrane sterol to 7.3 from 10.5 milligrams per gram of protein lowered the phase transition by as much as 17 degrees. The activation energies above and below the transition were similar in all cases.

F_1-ATPase is also sensitive to fatty acid composition. Haslam, Cobon, and Linnane[21] altered the unsaturated fatty acid composition of a fatty acid desaturase mutant between 20 and 80 percent unsaturation. The transition temperature shifted from 8°C when there was 83 percent unsaturation to 35°C when there was only 13 percent unsaturation. Activation energies above and below the transition temperature were similar at all levels of supplementation. Direct studies of the membrane using an electron paramagnetic resonance labeled probe showed a phase transition of the lipids at a temperature close to those reported for ATPase activity.

Both unsaturated fatty acid and sterol composition affect the activity of L-kynurenine-3-hydroxylase, an enzyme present in the outer mitochondrial membrane[22,23]. At concentrations up to 27 micrograms of sterol per milligram of mitochondrial protein,

ergosterol exhibited a stimulatory effect on enzyme activity. Higher levels diminished the activity. The transition temperature shifted from 11.1°C to 9.2°C when sterol was increased to 27 micrograms per milligram of protein. However, higher supplementation raised the transition back to 11.1°C. This effect may reflect a rearrangement of the lipid proximal to the enzyme causing a further conformation change.

The effect of altered unsaturated fatty acid and sterol species as well as altered sterol to phospholipid ratios on membrane properties has been studied extensively using artificial bilayers. The use of such systems simplifies interpretation of experimental results. Dipalmitoyl lecithin exhibits an endothermic transition from the gel to liquid-crystalline phase at 25°C. Differential scanning calorimetry shows the transitions as an increase in heat absorbed by the system. The heat absorbed at the transition varies inversely with increased addition of sterol until at 50 mole percent sterol the transition disappears[24]. An electron paramagnetic resonance study of this system similarly demonstrated an inverse relationship between the mobility of the phospholipid hydrocarbon chains and increasing sterol levels[25,26]. Changing the bilayer composition with the introduction of unsaturations in the hydrocarbon chain or modification of the chain lengths also affects the temperature of the phase transition, the heat absorbed at the transition and the mobility of the hydrocarbon chains[27,28]. The requirement of intrinsic enzymes for particular lipid species can be envisioned as necessary for modulation of the physical

properties of the membrane to provide an optimal environment for enzyme activity.

It is interesting to note that the enzymes studied, F_1-ATPase, cytochrome c oxidase, L-kynurenine-3-hydroxylase, and sterol methyltransferase, exhibit dissimilar transition temperatures which may reflect different sensitivities to the lipid environment. Some enzymes appear to function without a change in activation energy over a wide range of membrane fluidities where others function only within a narrow range. These differences may also reflect a heterogeneous lipid distribution in the membrane[3]. Clustering of lipid moieties to create some highly fluid regions and other less fluid regions has been demonstrated in model systems[13,27,29,30,31,32].

Ability to couple phosphorylation and respiration is related to the lipid composition of the mitochondrial membrane. The relationship between lipid composition and coupled respiration may be explained in part on the basis of altered enzyme activity. In addition, one must consider the permeability of the membrane. Mitchell and Moyle's chemiosmotic hypothesis[33] explaining the mechanism of oxidative phosphorylation requires a membrane impermeable to OH^- and H^+. Changes in lipid composition have been shown in model systems to alter membrane permeability to various solutes[34,35,36,37,38]. Therefore the ability to couple phosphorylation to respiration depends on the activity of the enzymes involved in respiration and phosphorylation as well as on the impermeability of the membrane to protons.

Proudlock, Haslam, and Linnane[39] used a mutant defective in unsaturated fatty acid synthesis to study the effects of altered fatty acid composition on aerobic yeast cultures. Growth on a respiratory substrate requires fully functional mitochondria, whereas growth on a fermentable substrate is independent of this requirement. Cells which have defective mitochondria are capable of growth on fermentable substrates but not on respiratory substrates. Comparison of growth on glycerol, a respiratory substrate, and glucose was shown to be proportional to the amount of unsaturated fatty acid supplemented. Under conditions of limiting unsaturated fatty acids, growth on glycerol ceased when the level of unsaturates dropped to 20 percent whereas growth on glucose did not stop until the unsaturates dropped to five percent. The increased growth on glucose indicated that unsaturated fatty acid depletion affected mitochondrial function. Glycerol-grown mitochondria from unsaturated fatty acid depleted cells exhibited normal morphology on examination by electron microscopy, normal levels of cytochromes and normal rates of respiration, suggesting that the lesion resulted from the inability to couple respiration to phosphorylation.

Further investigation by Haslam, Proudlock, and Linnane[40] showed that mitochondria from cells with only 15 to 20 percent unsaturated fatty acid exhibited no detectable phosphorylation activity or respiratory control. Normal malate and succinate dehydrogenase activities precluded impaired enzyme function. Mitochondria prepared from cells with 33 to 60 percent unsaturation showed a decreasing loss of

phosphorylation activity and respiratory control with the loss of unsaturated fatty acids. Addition of unsaturated fatty acids to the depleted cells reversed the effect. Uptake and incorporation of the unsaturated fatty acids into the membrane proceeded and restored phosphorylation until at 50 percent unsaturation, cell growth resumed. Protein synthesis was not required for the rejuvenation process, demonstrating that the loss of phosphorylation was due to altered lipid composition. Furthermore, comparison of whole cell lipids showed the sterol to phospholipid ratio changed under the condition of high or low fatty acid supplementation. This alteration of lipid ratios may indicate an important means of controlling the fluidity and permeability properties and hence the functionality of the membrane. The maintenance of such properties within a given range enables continued growth over a wide range of conditions and offers the organism a selective advantage.

The observation of diminished K^+ uptake by unsaturated fatty acid depleted mitochondria further suggested defective energy production. Intramitochondrial K^+ levels are maintained by an energy requiring transport process. Addition of ATP and valinomycin to supplemented mitochondria produces a rapid accumulation of K^+. However, unsaturated fatty acid depleted mitochondria lost K^+ on addition of valinomycin. The efflux demonstrated that the permeability of the membrane to K^+ had not been changed, and that the ability to generate energy for the transport process had been lost. The defect apparently arises from an increased permeability of the membrane to protons. Direct pH

measurement of respiratory competent mitochondria showed ejection of protons on addition of oxygen in the presence of a suitable respiratory substrate. Inclusion of the uncoupler carbonyl m-chlorophenylhydrazone before addition of oxygen abolished proton ejection. In this system, the behavior of unsaturated fatty acid depleted mitochondria was analogous to the uncoupled mitochondria. Passive proton permeability of the membranes was tested by challenging a nonrespiring mitochondrial suspension with a pulse of acid. Impermeability results in an abrupt decrease in pH followed by gradual alkalinization. The passive movement of H^+ across the membrane produces the latter process. Unsaturated fatty acid supplemented mitochondria exhibited a slow proton entry with a half-time of 160 seconds. Addition of carbonyl m-chlorophenylhydrazone reduced the half-time to 60 seconds. A further addition of valinomycin produced a half-time of 10 seconds. Unsaturated fatty acid depleted mitochondria did not exhibit as extreme a pH change with the acid pulse, and the half-time for proton entry was only 42 seconds. The alkalinization was unaffected by carbonyl m-chlorophenylhydrazone indicating that the membrane was already highly permeable to H^+. The altered proton permeability of the mitochondrial membrane prevents the establishment of a transmembrane proton gradient with the energy produced by the respiratory chain. Without the establishment of a transmembrane proton gradient, the electrochemical energy of the respiratory substrates cannot be converted into a usable form of energy.

Astin, Haslam, and Woods[41] isolated a mutant of Saccharomyces cerevisiae in which the sterol levels were manipulatable. A genetic lesion in porphyrin biosynthesis prevents the synthesis of components

requiring a haem-containing enzymes, e.g., methionine, unsaturated fatty acids and ergosterol. Addition of varying amounts of δ-amino-laevulenic acid to the growth medium reversed unsaturated fatty acid and sterol depletion of the cells, presumably by functioning as a suitable porphyrin intermediate. The effect of altered sterol levels alone was achieved by supplementation with excess unsaturated fatty acids. Depletion of unsaturated fatty acids and sterol resulted in a lowered growth yield, reflecting low mitochondrial phosphorylation efficiencies[42]. Mitochondria isolated from cells with the lowest unsaturated fatty acid and sterol content had a phosphorylation efficiency of only seven percent that of the wild-type parental. Under conditions where only sterol was limited, increased phosphorylation efficiencies were associated with increasing amounts of sterol. Proton permeability of nonrespiring mitochondria also paralleled sterol level. Whereas the fully supplemented mutant and wild type mitochondrial membranes were relatively impermeable over the temperature range of 6 to 30°C, the sterol depleted membranes were 14 times as permeable. The activation energy for proton entry also exhibited sterol dependence and was inversely related to the sterol content.

The evidence presented indicates a relationship of oxidative phosphorylation efficiency, and hence growth, with the physical state of the mitochondrial membranes. The physiologic effect is produced by diminution of sterol and unsaturated fatty acid levels. The similarity of respiratory rates where membrane composition is altered indicates that the enzymatic activities are not appreciably affected.

Instead, the modified composition produces a membrane with altered proton permeability. Under conditions of severe depletion the permeability is increased to the point where a trans-mitochondrial-membrane proton gradient can not be established to recover the energy produced by the respiratory chain. Hence, active transport and other energy requiring functions are not possible.

Sterols and membrane fluidity have also been implicated in membrane stretching capacity. Impermeable to sucrose and other disaccharides, intact mitochondria[43] and sphaeroplasts[44] behave as osmometers when suspended in a non-isotonic buffer. The response is monitored by a change in optical density, an increased optical density corresponding to a shrinking of the vesicle. Response to osmotic shock has been reported to reflect the fluidity of the plasma membrane[44,45,46,47]. Susceptibility of yeast sphaeroplasts to osmotic lysis corresponded to the degree of fatty acid unsaturation[44] indicating that increased fluidity decreased membrane stretching capacity. Hossack and Rose[45] investigated the effects of different sterols on sphaeroplast fragility. To alter the cellular sterol composition, anaerobic cultures were supplemented with a variety of sterols. On dilution of the sphaeroplasts into hypotonic medium, ergosterol and stigmasterol appeared to provide a more stable membrane as compared to campesterol, cholesterol, 7-dehydrocholesterol, 22,23-dihydrobrassicasterol, and β-sitosterol. Hossack and Rose[45] concluded that unsaturation at the C-22 position was the most important feature in conferring stability to the membrane and that

substitution of alkyl groups at C-24 and unsaturation in the sterol nucleus were unimportant.

However, growth experiments by Nes et al.[48] presented evidence indicating that the unsaturations at C-5, C-22, and C-27, as well as the C-24 methyl group of ergosterol are functionally significant. Supplementation of anaerobic cultures with unsaturated fatty acids and ergosterol sustained a normal cell yield. No enhancement of growth was observed on substitution of ergosterol with cholesterol. Hence Saccharomyces cerevisiae is apparently capable of discriminating between ergosterol and cholesterol. The preferential uptake of the former attaches a physiologic significance to the Δ-5,7 and Δ-22 unsaturations and the C-24 β-methyl group. Hossack and Rose[45] also observed that the cells discriminated among the sterols supplemented and enrichment varied between 67 and 93 percent. Ergosterol was preferred. Therefore the observation on resistance to osmotic swelling reported may reflect quantitative as well as qualitative differences in the sterol composition. Furthermore, the sterols used in the study do not present a systematic comparison of functionally significant parts of the molecule, there being multiple modifications in the sterol structures compared.

This thesis pursues further the effect of qualitative changes in sterol composition on membranes and discusses the effects in relation to mitochondrial function and overall impact on the cell. The study of an artificial membrane system is included to assay the functionally important moieties of the sterol molecule. The effect of sterol alteration on enzymatic activity, permeability, and structural rigidity is investigated.

III. Materials and Methods

A. Organisms and Culture Conditions

The following strains of Saccharomyces cerevisiae were used in this study: 3701b (H. Roman), a haploid uracil auxotroph; 3701b-n3 (this laboratory), a nystatin-resistant mutant clone isogenic to 3701b; Z008 (L. Miller), a nonsporulating diploid $his^{+/-}$ $try^{+/-}$; 8R1 (L. Miller) a nystatin-resistant mutant clone isogenic to Z008; S288c (S. Fogel) a haploid wild type; and MCC (active culture collection at Oregon State University), a diploid wild type. The predominant sterol found in 3701b, MCC, and S288c is ergosterol. 3701b-n3 contains predominantly ergosta-7,22-diene-3 β-ol[49]. Ergosterol is the dominant sterol in Z008 although significant amounts of ergosta-5,7,22,24(28)-tetraene-3 β-ol are accumulated[50]. 8R1 accumulates zymosterol as well as cholesta-5,7,22,24-tetraene-3 β-ol[50].

The organisms were grown routinely with vigorous shaking in broth containing one percent tryptone, 0.5 percent yeast extract and two percent ethanol. Temperature was maintained at 28°C. Stock cultures were stored at 4°C on slants containing one percent tryptone, 0.5 percent yeast extract, two percent glucose and 1.5 percent agar.

B. Preparation of Mitochondria

Mitochondria were prepared following a modification of the procedure described by Astin and Haslam[42]. Cells were washed once with distilled water, resuspended on 0.5 M β-mercaptoethanaol, 0.1 M tris-

(hydroxymethyl) aminomethane(TRIS) at a pH of 9.2, at two milliliters per gram wet weight of cells and incubated at 28-30°C for five minutes with gentle shaking. The cells were centrifuged at 12,100 x g and washed twice with a pH 5.8 solution of 0.7 M sorbitol, 0.3 M mannitol, 0.01 M citric acid, 0.01 M potassium phosphate, and 0.001 M ethylenediaminetetraacetic acid (EDTA). The pellet was resuspended in a ratio of one gram wet weight of cells to 2.5 milliliters of glusulase solution. This solution was made by mixing one part of a commercially available glusulase preparation (Endo Laboratories, Inc.) with four parts of the above buffer. Alternatively the glusulase was precipitated with ammonium sulfate as described by Schatz and Kovac[51] and resuspended at a concentration of approximately 900 units of sulfatase activity per milliliter of buffer. The glusulase-cell suspension was incubated at 28-30°C for one hour with gentle shaking and then centrifuged at 3,050 x g for ten minutes. The supernatant containing the glusulase was spun at 12,100 x g for ten minutes to remove cellular debris and stored at 4°C for reuse. The sphaeroplast pellet was washed twice with a 0.9 M sorbitol, 0.01 M Tris, 0.0005 M EDTA (pH 7.4) solution and resuspended in a minimum volume of this buffer. This suspension served as the sphaeroplast preparation in the shrinking and swelling experiments described below. To isolate mitochondria, the suspension was disrupted in a French pressure cell at 1-2000 p.s.i. Intact cells and cellular debris were removed by three centrifugations at 3,050 x g for ten minutes. The resulting supernatant was spun at 12,100 x g for 20 minutes to recover mitochondria. The pellet was washed once

with the same buffer, centrifuged at 12,100 x g for 20 minutes and resuspended. Centrifugation of the suspension at 3,050 x g for ten minutes removed the last of the cellular debris. The supernatant was spun at 12,100 x g for 20 minutes to yield the final mitochondrial preparation. Unless specified, all manipulations were done at 4°C.

In studies where intact, respiratory-competent mitochondria were not required the cells were washed once with distilled water, once with 0.9 M sorbitol, 0.01 M Tris, 0.0005 M EDTA, (pH 7.4) and resuspended in this buffer at a concentration of one gram wet weight of cells per milliliter. All following manipulations were done at 4°C. The cells were broken with a 30 second burst of a Bronwill MSK tissue homogenizer using 0.25 mm glass beads as described by Thompson and Parks[52]. The beads were allowed to settle out and the supernatant was centrifuged for ten mintues at 3,050 x g. The pellet was resuspended, broken again, centrifuged, and the supernatants combined. Cellular debris was removed and mitochondria recovered as described above.

When gradient purified mitochondria were required, the mitochondrial pellet was resuspended in a minimum volume of buffer and layered on a 20-70 percent sucrose gradient. The gradient also contained 0.05 M Tris, (pH 7.9). Centrifugation was for three hours at 60,000 x g and 5°C. The gradients were fractionated by punching a hole in the bottom of the tube and collecting the mitochondrial band.

For the anisotropy measurement, five milliliters of a mitochondrial suspension at an optical density of 0.6 at 450 nm was diluted with an

equal volume of probe solution. Probe solution was prepared by adding 0.1 milliliter of 0.002 M 1,6-diphenyl-1,3,5-hexatriene in tetrahydrofuran to 100 milliliters of 0.6 M mannitol, 0.0001 M EDTA, 0.01 M phosphate, (pH 6.6) with vigorous stirring.

C. Determination of Respiratory Competency

ADP-coupled oxidative phosphorylation was determined polarimetrically using a Gilson oxygraph equipped with a Clarke electrode. A circulating water bath surrounding the chamber controlled the temperature. Measurements were made at 17°C, 27°C, and 35°C. The chamber contained in 1.8 milliliters: 0.9 M sorbitol, 0.0012 M EDTA, 0.006 M K_2HPO_4, 0.006 M $MgCl_2$, 0.024 M Tris, 0.024 M maleate, 0.2 percent bovine serum albumin, (pH 6.6). Ethanol at a final concentration of 0.5 percent served as the respiratory substrate. Other additions where indicated resulted in a final concentration of 5.1×10^{-6} M ADP, 5×10^{-5} M KCN, 5×10^{-5} M carbonyl cyanide-m-chlorophenylhydrazone.

D. Shrinking and Swelling of Mitochondria and Sphaeroplasts

Enzymatically prepared mitochondria were prepared as described above and were diluted into concentrations of sucrose ranging from 0.0 to 1.0 molar. These solutions also contained 0.01 M Tris, (pH 7.4). Following incubation at room temperature for 30 minutes the absorption at 520 nm was determined. Sphaeroplasts were prepared as described above, resuspended in 0.7 M sorbitol, 0.3 M mannitol, 0.1 M citric acid, 0.01 M potassium phosphate, 0.01 M EDTA

(pH 7.4) and diluted into the varying concentrations of sucrose as well as varying concentrations of sorbitol. The optical density at 600 nm was determined after incubation for 30 minutes at room temperature.

E. L-kynurenine-3-hydroxylase Activity

L-kynurenine-3-hydroxylase activity was determined by modification of the method of Schott et al.[53]. Assay mixtures were 1.4×10^{-4} M NADPH, 9.5×10^{-3} M potassium cyanide, 4.8×10^{-5} M $MgCl_2$, 3.8×10^{-5} M p-chloromercuribenzoic acid, 9.5×10^{-4} M L-kynurenine, 0.005 M Tris, (pH 7.9). Reactions were started with the addition of 50 microliters of a mitochondrial preparation at a concentration of about 60 milligrams of protein per milliliter and were carried out over the range of temperatures 10°C to 40°C in a Scientific Industries temperature gradient incubator. Gentle shaking provided maximal aeration. After 40 minutes the reactions were terminated by the addition of 0.5 milliliters of 0.2 M $K_3(Fe(CN)_6)$ in 1 M phosphate buffer, (pH 6.8). Following reduction by the addition of 100 milligrams of ascorbic acid, the solution was extracted immediately with 1.5 milliliters of butanol-HCl (12 milliliters of butanol mixed with one milliliter of concentrated HCl), and then saturated with NaCl. The mixture was centrifuged at 500 x g at room temperature in an International centrifuge. The optical density of the organic phase was determined at 492 nm using a Zeiss PMQ II spectrophotometer. A blank was prepared under the

same conditions by omitting L-kynurenine during the incubation period. Kynurenine was added to the blank following termination with $K_3(Fe(CN)_6)$ solution. All values are an average of three experiments.

F. Preparation of Liposomes with Polarization Probe

L-γ-phosphatidylcholine (type III-E from egg yolk) and sterol, when desired, were dissolved in chloroform at a concentration at one milligram of sterol when used to eight milligrams of phospholipid. The solution was evaporated to dryness under a stream of argon. Addition of an aqueous 0.05 M KCl solution resulted in a final concentration of 0.8 milligrams of phosphatidylcholine per milliliter of solution which was stirred vigorously for one minute. An equal volume of fluorescence probe solution, made by adding 0.1 milliliter of 0.002 M 1,6-diphenyl-1,3,5-hexatriene in tetrahydrofuran to 100 milliliters of 0.02 M potassium phosphate, (pH 6.5), was added. Centrifugation at 500 x g yielded a slightly turbid supernatant containing the liposomes.

G. Fluorescence Measurement

Following labelling of the sample with the fluorescent probe as described above, fluorescence polarization was measured by the instrument described by Ayres et al.[54] over the temperature range of 15°C to 40°C. 1,6-diphenyl-1,3,5-hexatriene was excited at 363 nm. Fluorescence emission at 460 nm was detected on both sides of the sample at 90° through 3-74 Corning glass filters to eliminate

wavelengths below 390 nm. The measurement of fluorescence intensities through a polarizer oriented parallel or perpendicular to the direction of the polarization of the excited beam, $I_{\parallel}$ and $I_{\perp}$, enabled the calculation of fluorescence anisotropy from the relationship:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

The calculated anisotropy is the average of 3,000 measurements of $I_{\parallel}$ and $I_{\perp}$.

H. Methyltriphenylphosphonium Ion Uptake

Uptake of the lipophilic probe, methyltriphenylphosphonium bromide, was determined at 17°C, 27°C, and 35°C. The reaction mixture contained in 0.5 milliliter: 0.9 M sorbitol, 0.0012 M EDTA, 0.006 M H_3PO_4 0.006 M $MgCl_2$, 0.024 M Tris, 0.024 M maleate, 0.2 percent bovine serum albumin, (pH 6.6) and 2.0 percent ethanol. Fifty microliters of a mitochondrial suspension (0.5 to 1.5 milligrams of protein) were added to the mixture and preincubated for two minutes. Low protein levels were used to prevent anaerobiosis. Addition of 50 microliters of 2.8×10^{-3} M ^{14}C-methyltriphenylphosphonium bromide at a specific activity of 1.4 Ci per mole started the reaction. Aliquots were removed over a seven minute time course. Mitochondria were collected by filtration on Gelman cellulose acetate filters (0.05 µm pore size)

and washed with a three milliliter aliquot of ice cold buffer containing unlabelled methyltriphenylphosphonium bromide to remove any cation not entrapped by the mitochondria. The filters were dried and the retained radioactivity was determined by a Beckman LS8000 liquid scintillation counter using a toluene cocktail (3.0 grams 2,5-diphenyloxazole, 0.1 gram 1,4-bis-(2-(5-phenyloxazolyl))-benzene, 1.0 liter toluene). Other additions of oxidative phosphorylation uncouplers and inhibitors are noted in the text. Mitochondrial volumes were determined to be 3.1 microliters per milligram of mitochondrial protein using ^{14}C-sucrose and ^{3}H-H_2O as described by Perkins et al.[55]

I. Lipid Analyses

Determination of sterol to phospholipid ratios was accomplished by growing the organisms in broth containing one percent tryptone, 0.5 percent yeast extract, 0.1 percent sodium acetate, 0.2 microcuries per milliliter sodium ^{14}C-acetate, and two percent ethanol. The lipids were extracted with dimethylsulfoxide and chloroform as described by Taylor and Parks.[56] The resulting organic phase was evaporated to dryness under a stream of nitrogen and its components were separated by thin layer chromatography (silica gel 60 F-254, 0.25 mm thick, manufactured by E. M. Laboratories) using the solvent systems of Skipsky et al.[57] The resulting phospholipid bands were visualized using iodine vapor. Sterol and phospholipid bands were scraped and radioactivity determined using Aquasol II (New England

Nuclear) which was made five percent aqueous.

The numerous steps involved in cell fractionation precluded the use of this method for the determination of mitochondrial sterol to phospholipid ratios and necessitated the following procedure. Mitochondrial lipids were extracted following the procedure of Bligh and Dyer.[58] An aliquot was removed to determine the amount of phospholipid using the colorimetric phosphorous determination of Ames.[59] The remainder was evaporated to dryness with a stream of nitrogen and the free sterols isolated by thin layer chromatography. The free sterol band was batch eluted using eight washes of chloroform-methanol (4:1). Following evaporation to dryness, the organic material was dissolved in 100 microliters of pyridine and acetylated using acetic-^{3}H-anhydride at a specific activity of 244 millicuries per mole. The sensitivity of the acetic anhydride to moisture made it necessary to include a concentration curve using various amounts of cholesterol. The acetylation mixture was incubated overnight at room temperature. Following evaporation of the reactants, isolation of the labelled sterol acetates was achieved by thin layer chromatography using chloroform as the solvent. The sterol acetate bands were scraped and radioactivity determined using the above described toluene cocktail.

The fatty acid composition of the phospholipids was determined following extraction and separation of the lipid components as described. The phospholipid band was eluted with three successive washes of methanol. The samples were dried under a stream of nitrogen,

and then further dried in a vacuum dessicator for a minimum of 20 minutes. One milliliter of 0.5 M sodium methoxide in anhydrous methanol was added to each sample. After five minutes at room temperature, two milliliters of 2 N HCl were added, and the resulting fatty acid methyl esters were extracted using two washes of hexane-ether (1:1). The relative amounts of the fatty acids were determined by gas chromatography in a Hewlett Packard 5710 A gas chromatograph using a 1.83 meter by 1/8 inch stainless steel column containing 10 percent DEGS-PS as the liquid phase on an 80/100 Supelcoport support. The column temperature was maintained at 180°C with a gas flow rate of 50 milliliters of carrier gas per minute. Areas were determined as the product of the width at half height and the height of the peak.

Total sterol composition was determined by gas chromatography and ultraviolet absorption spectroscopy of the organic phase following saponification and hexane extraction. Saponification was conducted by refluxing the pelleted sample with two milliliters of 60 percent KOH, three milliliters of methanol, and two milliliters of 0.5 percent pyrogallol in methanol at 85°C. Gas chromatography was conducted in a Varian 2740 gas chromatograph interfaced with a CDS 111 data processor using a glass column (1.22 meters by 2 millimeters) containing 3 percent OV-17 as the liquid phase on H/P chromasorb W (100 to 120 mesh) support. Column temperature was maintained at 265°C with a gas flow rate of 20 milliliters per minute.

Ultraviolet absorption spectroscopy was conducted with a Cary model 11 recording spectrophotometer between 310 and 200 nm.

J. Protein Determiantion

Protein was determined according to the method of Lowry *et al.*[60] using bovine serum albumin as a standard.

IV. Results and Discussion

A. Lipid Analysis

Sterol to phospholipid ratio as well as fatty acid composition affect the properties of membranes. For this reason, these parameters for both the whole cell and mitochondrial lipids were determined.

The sterol to phospholipid ratio found in the whole cell and mitochondrial lipid fraction from the organisms studied are shown in Table 1. The whole cell ratios are determined by the amount of ^{14}C-acetate incorporated into the free sterol and phospholipid fractions as described in the Materials and Methods sections. Because of the heterogeneity in the phospholipid composition, no attempt was made to convert the data into a molar ratio. Therefore the data shown for the whole cell and mitochondrial sterol to phospholipid ratios can not be directly compared. An approximation for comparative purposes may be made on the basis that 28 carbons of ^{14}C-acetate are incorporated into each sterol molecule and 34 carbons of ^{14}C-acetate are incorporated into each phospholipid molecule. This conversion increases the sterol to phospholipid ratio reported on the basis of the ratio of radioactivity. Therefore, the data reported in Table 1 for the whole cell represents a lower limit for the value. Using this criterion of comparison, no enrichment of the mitochondrial membrane for sterol is observed.

TABLE I. RATIO OF STEROL TO PHOSPHOLIPID IN THE WHOLE CELL AND MITOCHONDRIAL LIPID FRACTION. Extraction and quantitation of sterol and phospholipid are described in Materials and Methods. The ratio for the whole cell lipid fraction is a ratio of the ^{14}C-counts in the free sterol fraction to the ^{14}C-counts in the phospholipid fraction. The mitochondrial sterol to phospholipid relationship is expressed as a molar ratio of free sterol to phospholipid. Cells analyzed during logarithmic growth were harvested after 14 hours of growth (between 150 and 200 Klett) and during stationary growth were harvested after 40 hours.

Strain	Growth Phase	Sterol to Phospholipid Ratio	
		Whole cell	Mitochondrial
S288c	Logarithmic	0.10 ± 0.02	0.15 ± 0.02
	Stationary	0.17 ± 0.02	
3701b	Logarithmic	0.20 ± 0.02	0.096 ± 0.005
	Stationary	0.14 ± 0.02	
3701b-n3	Logarithmic	0.20 ± 0.02	0.11 ± 0.02
	Stationary	0.16 ± 0.02	
Z008	Logarithmic	0.17 ± 0.01	0.105 ± 0.005
	Stationary	0.16 ± 0.01	
8R1	Logarithmic	0.17 ± 0.02	0.12 ± 0.03
	Stationary	0.17 ± 0.04	
MCC	Logarithmic	0.13 ± 0.01	0.19 ± 0.05
	Stationary	0.15 ± 0.02	

The cellular sterol to phospholipid ratio remained constant throughout the growth of 8R1 and Z008, whereas a decrease in 3701b and 3701b-n3 and an increase in S288c and MCC were observed. Although considerable variation is seen during logarithmic growth, ranging from 0.10 in S288c to 0.20 in 3701b and 3701b-n3, the ratios in all the organisms were the same during the stationary phase. The significance of the variability of the sterol to phospholipid ratio with growth phase is unclear, but does appear to be strain related. Whereas the sterol wild types show logarithmic values of 0.10 for S288c, 0.20 for 3701b and 0.13 for MCC, no variation is seen in the two sets of isogenic strains.

The mitochondrial sterol to phospholipid ratios were determined only during logarithmic growth. With the exception of the kynurenine hydroxylase enzyme assay, all of the mitochondrial isolations were prepared from cells in logarithmic growth phase. Because of the numerous manipulations in the quantitation of the mitochondrial sterols, a lipid extract labelled with ^{14}C-acetate as described in Materials and Methods and quantitated for sterol and phospholipid composition using the procedure outlined for the mitochondrial determinations was used to determine loss of material, if any. A loss of 22 percent of the ^{14}C-counts was observed. The data reported in Table I for the mitochondrial sterol to phospholipid ratio has been corrected for the loss of sterol. A range of sterol to phospholipid ratios are observed in the organisms studied, but again the ratios for the two sets of isogenic strains are the same.

The fatty acid composition of the phospholipids isolated from a whole cell lipid extraction of the various strains is shown in Table II. The dominant species are palmitoleate (16:1) and oleate (18:1) with lesser amounts of palmitate (16:0) and stearate (18:0). No significant differences in percent composition are observed between the strains or with respect to growth phase.

In most cases, the percent composition of the fatty acid species present in the mitochondrial phospholipid fraction was different from the cellular composition. With the exception of Z008 and 8R1, there is an increase in the percent of the 18:1 species with a concomitant decrease in the 16:1 species, the total percent unsaturation remaining constant in all cases. The difference in the amount of 18:1 and 16:1 species was not observed in 8R1 or Z008, the mitochondrial composition paralleling that of the whole cell.

The observed differences seen in the composition of the phospholipid fatty acids was not expected. Haslam et al.[40] reported similarities in the fatty acid composition of the mitochondrial lipids and the lipids of the whole cell from which the mitochondria were isolated. The data presented here indicates that the mitochondrial lipid components, in particular the phospholipid fatty acid constituents, do not necessarily parallel the whole cell lipid composition and appear to be a property of the strain studied.

To investigate further the concept of differential lipid distribution in the cell, 3701b was grown in the presence of 15-aza-24-methylene-D-homocholestadiene (azasterol), a potent inhibitor of fungal growth at very low concentrations. Hays et al.[61]

TABLE II. PERCENT COMPOSITION OF FATTY ACID COMPONENTS OF THE CELLULAR AND MITOCHONDRIAL PHOSPHOLIPIDS. Extraction and quantitation of the phospholipid fatty acid moieties is described in Materials and Methods. Cells analyzed during logarithmic growth were harvested after 14 hours of growth and during stationary growth were harvested after 48 hours of growth. In the table below, standard deviations are indicated. S represents analysis during stationary phase; L during logarithmic phase. Whole cell composition is noted as WC; mitochondrial as M.

Strain	Growth Phase	Source	% Composition				% Total Unsaturation
			16:0	16:1	18:0	18:1	
8R1	S	WC	9.5 ± 1.2	48.1 ± 1.6	4.9 ± 0.7	36.6 ± 3.4	84.7
		M	8.3 ± 1.1	48.2 ± 1.1	4.1 ± 0.4	39.6 ± 2.6	87.8
	L	WC	8.9 ± 0.6	47.2 ± 5.8	4.5 ± 1.0	39.2 ± 5.0	86.4
		M	6.0 ± 0.9	49.4 ± 0.8	3.8 ± 1.3	42.0 ± 0.2	91.4
Z008	S	WC	9.1 ± 0.3	47.3 ± 3.1	4.6 ± 1.1	40.9 ± 0.6	88.2
		M	11.2 ± 2.1	40.4 ± 1.3	5.0 ± 0.1	43.5 ± 0.8	83.9
	L	WC	8.2 ± 1.2	48.2 ± 4.7	5.0 ± 0.1	38.6 ± 3.3	86.8
		M	11.5 ± 2.1	40.8 ± 7.7	5.4 ± 0.7	44.5 ± 4.0	85.3
S288c	S	WC	12.5 ± 0.5	51.3 ± 2.2	4.3 ± 9.0	31.8 ± 1.9	83.1
		M	12.1 ± 2.0	36.2 ± 6.2	7.1 ± 1.1	44.6 ± 5.5	80.8
	L	WC	11.3 ± 1.0	55.3 ± 1.9	3.2 ± 0.6	29.5 ± 1.4	84.8
		M	10.9	47.9 ± 0.3	2.9 ± 0.4	38.2 ± 0.3	86.1
MCC	S	WC	10.5 ± 0.1	46.3 ± 6.9	5.8 ± 0.8	38.0 ± 8.2	84.3
	L	WC	9.9 ± 1.2	52.8 ± 2.5	4.5 ± 2.5	32.6 ± 0.8	85.4
		M	6.5 ± 0.5	49.1 ± 9.0	4.4 ± 1.1	40.1 ± 8.4	89.2
3701b	S	WC	11.5 ± 2.2	49.1 ± 5.6	6.1 ± 1.4	32.7 ± 5.6	81.8
		M	5.3*	35.0*	3.9*	55.8*	90.8
	S	WC	8.7 ± 0.8	50.7 ± 8.1	4.5 ± 1.1	36.4 ± 7.5	86.6
		M	6.7 ± 0.7	48.5 ± 1.7	3.6 ± 0.2	40.9 ± 1.0	89.4
3701b-n3	S	WC	9.1 ± 1.3	55.9 ± 4.8	4.3 ± 0.8	30.5 ± 5.6	86.5
		M	7.7 ± 2.3	42.4 ± 3.8	5.6 ± 0.1	44.4 ± 6.3	86.8
	L	WC	8.3 ± 2.2	55.6 ± 2.0	4.9 ± 0.7	31.1 ± 4.3	86.7
		M	5.8 ± 0.9	58.8 ± 1.8	2.1 ± 0.1	34.3 ± 1.1	93.0

* - Only one sample

reported the accumulation of ergosta-8,14-diene-3β-ol, given the trivial name ignosterol, in place of ergosterol when a wild type strain was grown in the presence of azasterol at concentrations below the inhibitory level. As the concentration of azasterol in the medium was increased, the amount of ergosterol decreased and the amount of ignosterol increased. This change in sterol composition is easily monitored in ultraviolet profiles of the extracted lipid. Ergosterol has an absorption due to the Δ5,7-conjugated diene at 272 and 282 nm, whereas the Δ 8,14-heteroannular conjugated diene of ignosterol exhibits a λ_{max} at 250 nm. To determine if ergosterol was prefenentially incorporated into the mitochondrial membrane when both ergosterol and ignosterol were present in the cell, the non-saponifiable lipids of the whole cell and of gradient purified mitochondria were examined following growth for 36 hours in the presence of 5 nanograms of azasterol per milliliter of culture medium. The resulting ultraviolet profile is shown in Figure 1. From the extinction coefficients (ε18,000, λ_{max} 282 nm for ignosterol; ε11,900, λ_{max} 282 nm for ergosterol)[61] the percent of the sterol species may be calculated. Whereas the whole cell fraction is composed of 42 percent ignosterol and 58 percent ergosterol, the mitochondria are virtually devoid of ignosterol. These data suggest a selective deposition of sterol in the mitochondria. This suggestion is corroborated by a report of Gordon and Stewart[62] in which anaerobic yeast cells supplemented with cholesterol replaced the cholesterol in the mitochondrial membrane with ergosterol on aeration. Ergosterol

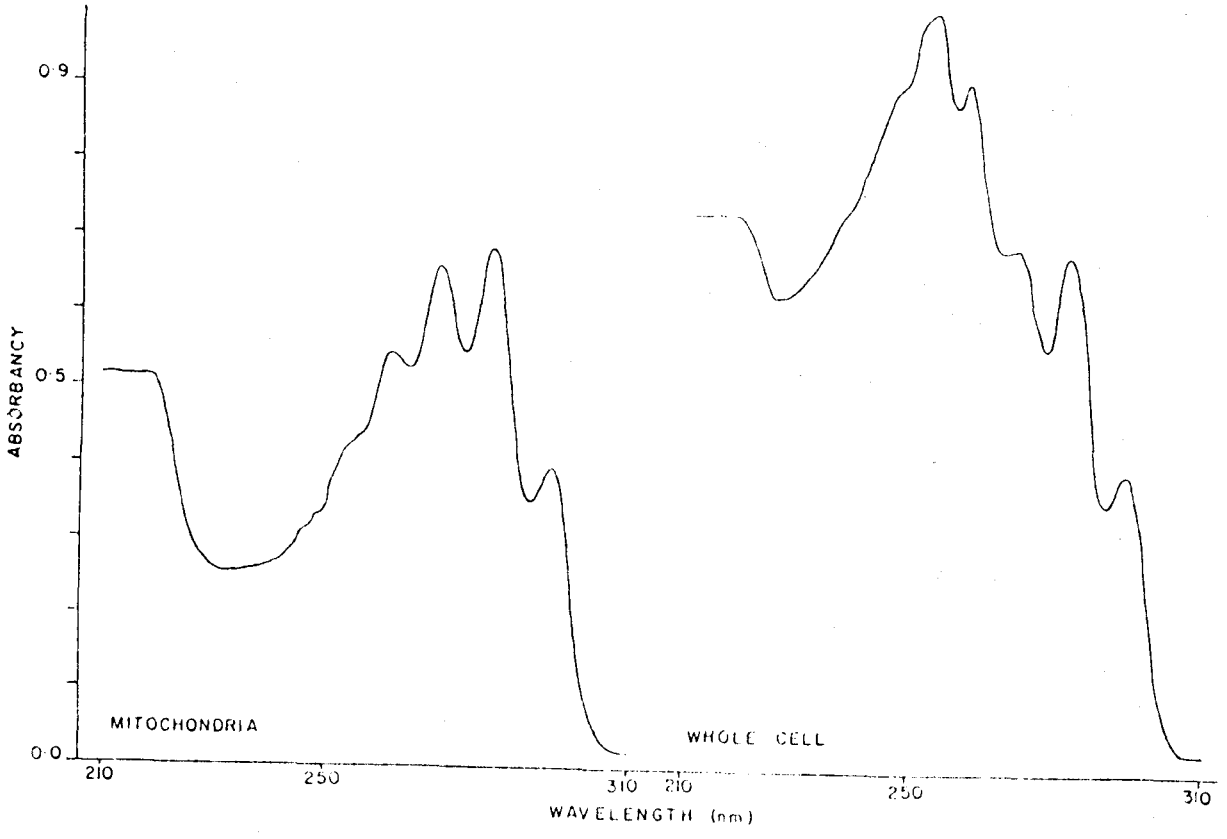

FIGURE 1. ULTRAVIOLET PROFILE OF CELLULAR AND MITOCHONDRIAL NON-SAPONIFIABLE LIPIDS. The experiment is described in the text.

appears to be the preferred sterol for deposition in the mitochondrial membrane.

B. Fluorescence Polarization Measurements

From the variety of methods available to study the physical structure of membranes and lipid bilayers, fluorescence polarization techniques were selected to investigate the physical properties of the mitochondrial membrane. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene was selected for use in this system for a number of reasons. Unlike the spin labelled fatty acids used in electron paramagnetic resonance studies, 1,6-diphenyl-1,3,5-hexatriene is relatively small. Small size together with a high extinction coefficient and high fluorescence quantum yield enable its use at very low concentrations. This feature reduces the effect of the probe on the bilayer[63]. It was desirable to introduce the probe to the mitochondrial bilayer in vitro so that problems arising from differential uptake, distribution, and metabolism of the probe could be avoided. The 1,6-diphenyl-1,3,5-hexatriene molecule will not fluoresce unless embedded in a hydrophobic region. Therefore, probe which had not entered the bilayer will not contribute to the measured anisotropy. The emission and absorption bands of 1,6-diphenyl-1,3,5-hexatriene are well separated, minimizing the contributions of scattered excitation light due to energy transfer from one probe molecule to another. Absorption and fluorescence transition moments are oriented along the major axis of the molecule so that the degree of fluorescence polarization relates to the

viscosity opposing rotation of the long axis of the molecule[64]. Therefore, high anisotropy values indicate a viscous environment where rotation of the probe is hindered.

The data are reported here as anisotropy rather than in terms of microviscosity. The microviscosity calculation for lipid bilayers as developed by Shinitsky et al.[65] arises from the comparison of bilayer or membrane anisotropy to the anisotropic behavior of the fluorescent probe solubilized in paraffin oils. Criticism of this transformation has arisen with the observation that the anisotropic behavior of various probe molecules, including 1,6-diphenyl-1,3,5-hexatriene, is dependent on the type of paraffin oil used in the reference measurement[66]. Since the observations presented in this thesis are of a comparative nature, it seemed judicious to avoid the controversy over the legitimacy of the microviscosity calculation by reporting the results in terms of anisotropy.

Since the anisotropy measurement of the mitochondria isolated from the various strains were not made simultaneously because of the complexity of the isolation procedure, it was necessary to establish the reproducibility of mitochondrial isolation and anisotropy measurements to ensure that observed differences were attributable to changes in membrane properties rather than to variations in experimental procedure. The reproducibility of this technique is shown in Figure II. Two independent mitochondrial preparations from 3701b on different days yielded the Arrhenius curves shown. The close agreement of anisotropy values indicates consistency

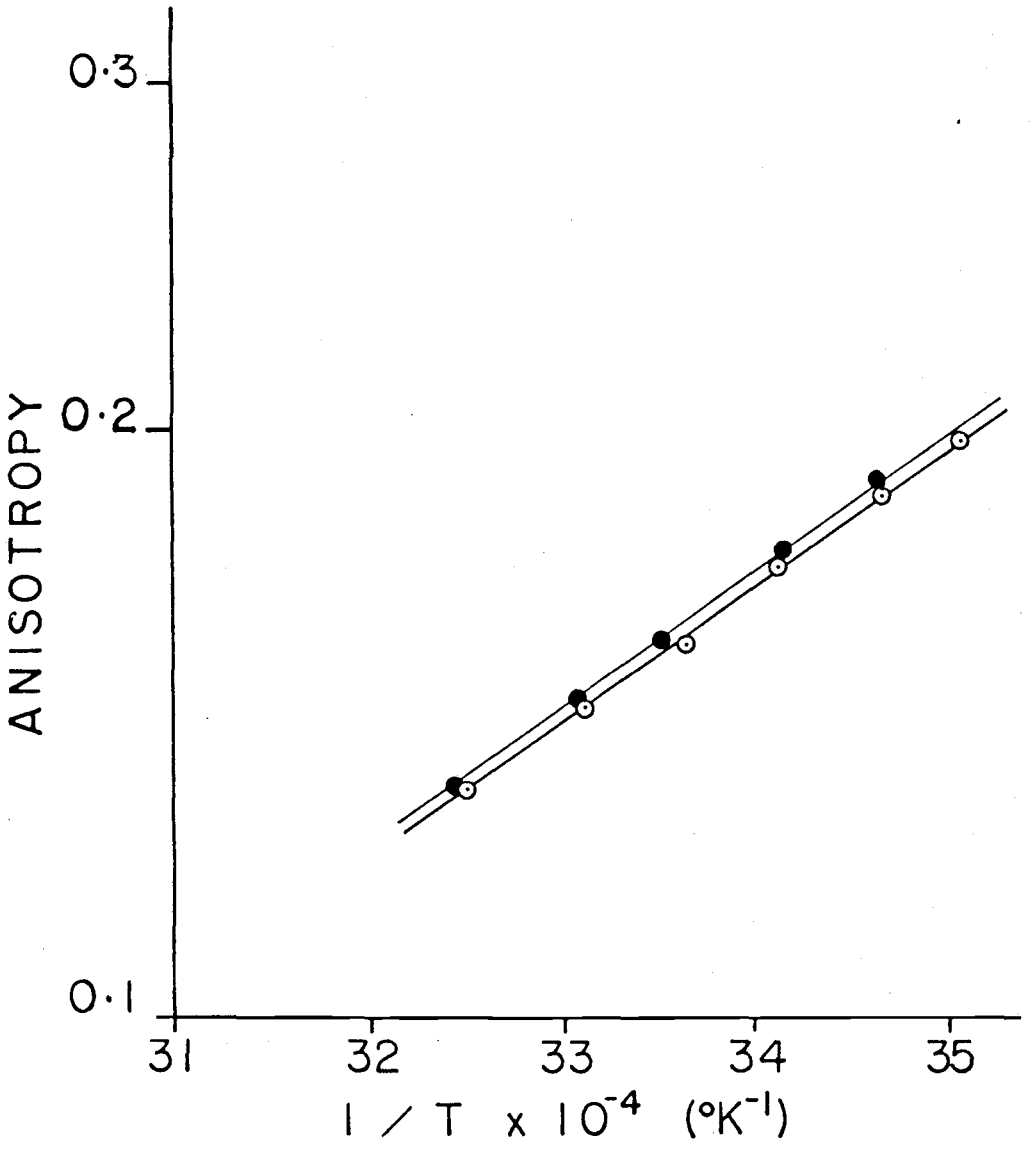

FIGURE 11. REPRODUCIBILITY OF FLUORESCENCE MEASUREMENT. Mitochondria were isolated from 3701b as described in Materials and Methods on different days.

in the isolation of the mitochondria as well as reproducibility in the anisotropy measurement.

The occurence of ergosterol as the major sterol in yeast suggested a role for this sterol as a modulator of membrane properties. For this reason, the mobility of the probe in mitochondrial membranes isolated from a number of wild types was expected to be similar. However, Figure III shows a variation in the anisotropy values. Although measurements from S288c and 3701b are almost identical, MCC anisotropy values are significantly elevated. As noted above, the fatty acid composition of the phospholipid fraction is similar in all of the strains. The high anisotropy values of the MCC membrane may reflect the difference in the sterol to phospholipid ratio. This study did not determine the composition of the various phospholipid species in the strains used. The superposition of the anisotropy data from S288c and 3701b, which show different sterol to phospholipid ratios, suggests a modulation of fluidity by differences in phospholipid head group composition. Preliminary evidence indicates significant variability in the ratio of phosphatidylcholine and phosphatidyl insitol in these strains[67]. Studies in artificial membrane systems have shown that sterol addition to bilayers composed of various phospholipid species reduced the bilayer fluidity as measured by electron paramagnetic resonance and nuclear magnetic resonance[68,69,70]. This effect was reported in all phospholipid species studied, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, ceramide and sphingomyelin, with the exception

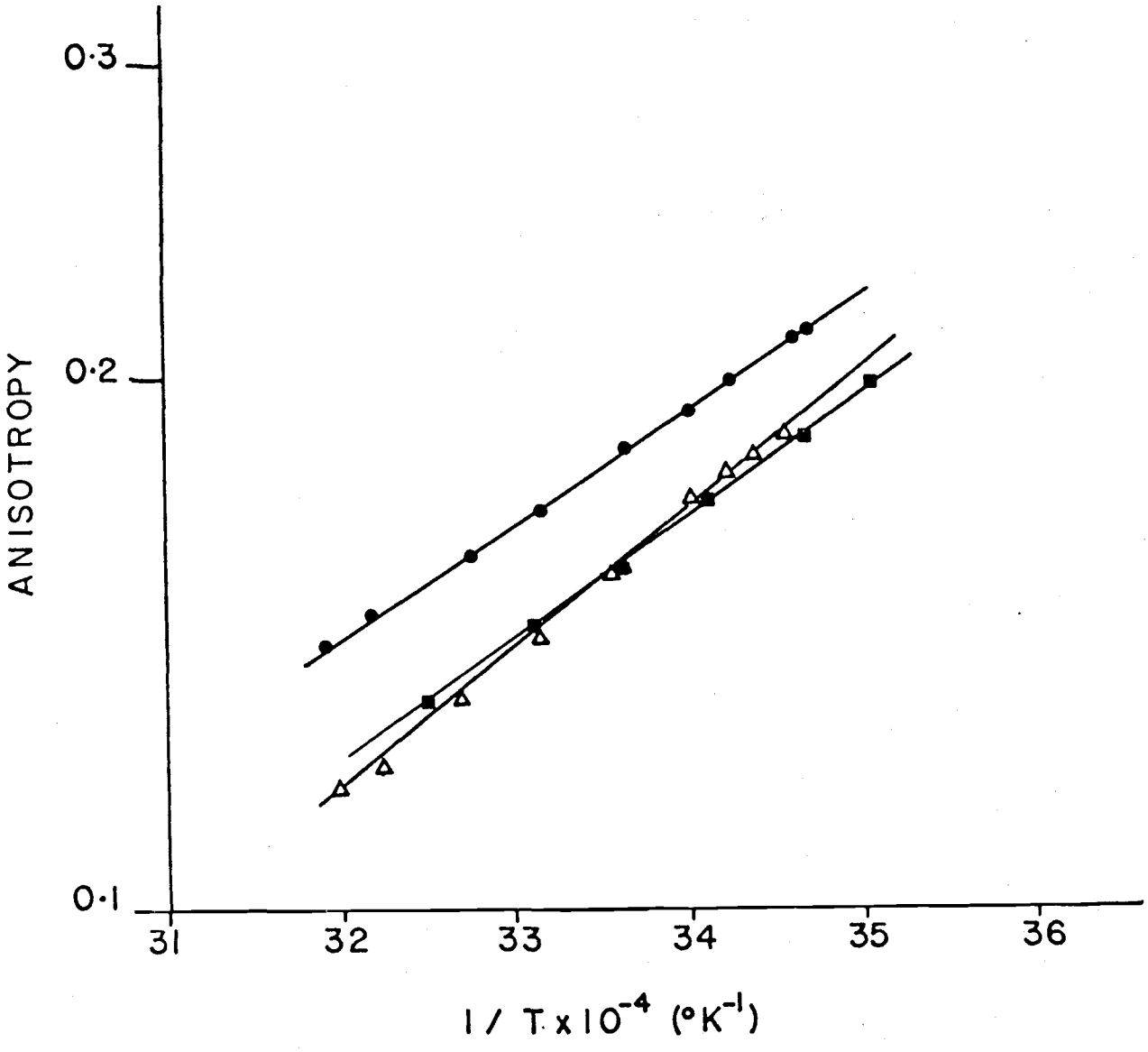

FIGURE III. ARRHENIUS CURVES OF ANISOTROPY OF A FLUORESCENT PROBE EMBEDDED IN MITOCHONDRIAL MEMBRANES ISOLATED FROM STEROL WILD-TYPE STRAINS. Mitochondria were isolated and labelled with the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene as described in Materials and Methods. ●--MCC; ■--3701b; △--S288c.

of cardiolipin. Differential scanning calorimetric profiles showed that in cases where a phase transition characteristic of the phospholipid existed, as in the cases of sphingomyelin and cerebroside, addition of sterol eliminated the transition[69]. Furthermore, Trauble and Eibl's[71] examination of artificial phosphatidic acid bilayers demonstrated the sensitivity of the lipid bilayer to the charge present in the phospholipid head group. An increase in pH from seven to nine increased the charge on the phosphatidic acid molecules and lowered the transition by 20°C. The divalent cations Mg^{+2} and Ca^{+2} increased the temperature of the phase transition by neutralizing the charge on the phosphatidic acid. Reiber's study[72] of cholesterol-phospholipid interactions further suggested that the membrane polar groups, including the phospholipid headgroup ester carbonyl and the 3β-hydroxyl of the sterol, by virtue of their hydration properties, altered the aqueous region of the membrane surface. This is discussed in more detail below. Differences in phospholipid species may mask the effect of altered sterol to phospholipid ratios in 3701b and S288c.

Whereas no phase change was observed in the mitochondria of the wild types, Arrhenius curves of anisotropy from the mutant strains all exhibit discontinuities. See Figure IV. Although the major sterol accumulated by Z008 is ergosterol, a transition at 23.5°C is observed. This discontinuity is believed to result from the presence of significant levels of ergosta-5,7,22,24(28)-tetraene-3β-ol. 8R1 similarly exhibits a transition at 23.3°C as well as reduced anisotropy values. On the basis of molecular model building,

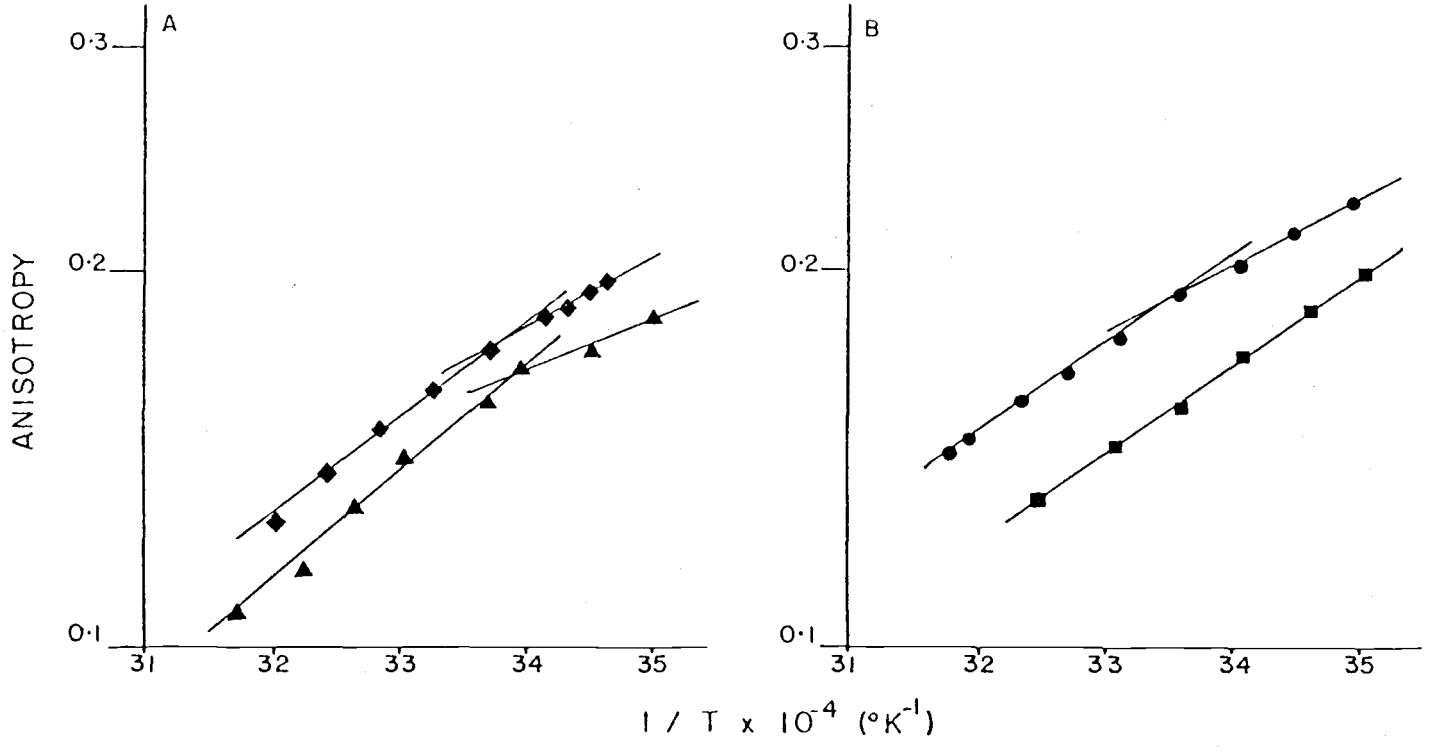

FIGURE IV. ARRHENIUS CURVES OF ANISOTROPY OF A FLUORESCENT PROBE EMBEDDED IN MITOCHONDRIAL MEMBRANES ISOLATED FROM STEROL MUTANT STRAINS. Mitochondria were isolated and anisotropy measured as described in Materials and Methods. Figure 4A shows curves from Z008--▲, and its isogenic 8R1--◆. Figure 4B shows curves from 3701b--■, and its isogenic 3701b-n3--●.

the observed increase in fluidity is consistent with the anticipated effect of substituting zymosterol for ergosterol. The unsaturation at the C-8 position of zymosterol pulls the C and D rings of the sterol molecule further out of the A-B plane than does the Δ5,7-unsaturation of ergosterol. (See Appendix I for sterol numbering system.) Such a modification does not allow close packing of the phospholipid molecules in the bilayer, hence providing more room for movement of the hydrocarbon chains. Artificial membrane studies indicate that the maximal orientation of the phospholipid fatty acyl chains occurs when the addition of sterol to the bilayer decreases the molecular area at the bilayer surface. This condensing effect varies with sterol structure as well as with the species present in the fatty acyl chains. The non-planar nucleus can be envisioned as increasing the volume of the cavity required to accomodate the sterol in the bilayer. Such sterol interactions are shown schematically in Figure V. This model predicts that those sterols possessing a planar nuclear ring structure without bulky substituents of the side chain will permit a closer association of the lipid moieties, thereby hindering movement of the hydrocarbon chains and resulting in decreased fluidity. Also shown in Figure IV is a comparison of the anisotropy data from the mitochondrial membranes of 3701b-n3 and 3701b. In this case, the Δ7-unsaturation produces a more planar sterol molecular than the Δ5,7-unsaturation of ergosterol. A transition occurs at 24.6°C, the elevated anisotropy values indicating a less fluid membrane.

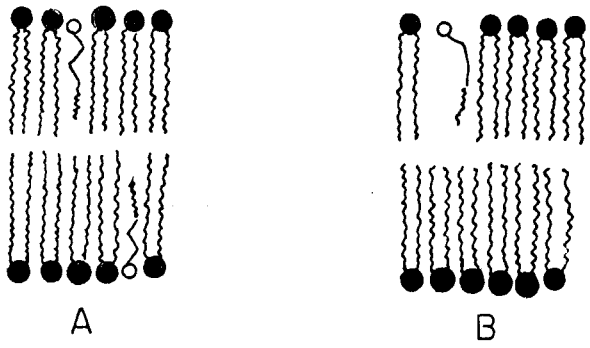

FIGURE V. SCHEMATIC DIAGRAM OF STEROL-PHOSPHOLIPID INTERACTIONS. The phospholipid polar head group is denoted by the solid spheres, the open circles representing the sterol 3β-hydroxyl group. Acyl chains are denoted by the wavy lines, the straight lines representing the rigid sterol nucleus. Case A shows the interaction of a planar sterol molecule in a phospholipid bilayer. Case B demonstrates the interaction of a nonplanar sterol molecule with the bilayer.

In this system, Arrhenius activation energies are not calculated because the fluorescence emission has not been quantitated on a molar basis. However, it is of interest to note that in both cases of the parental strain and its isogenic mutant, the slopes of the lines at temperatures above the transition are nearly identical. These data indicate that the substitution of a sterol other than ergosterol into the mitochondrial membrane of yeast affected the physical properties of the membrane. The observed change in anisotropy is consistent with predictions made from molecular model building.

To determine if the observations made in the biological system were consistent with those reported for electron paramagnetic resonance, monolayer, permeability, and differential scanning calorimetry studies, the fluorescence polarization experiments were extended

to include the study of an artificial membrane system. The careful selection of sterols for use in this study would also provide information on the structurally important portions of the sterol molecule. The artificial system contained various sterols and egg lecithin as a source of phospholipid.

The lipids were initially mixed together in a ratio of one milligram of sterol to eight milligrams of egg lecithin. Because of reported differences in solubility of different sterols in phospholipids[73] the vesicle preparations were extracted and the sterol to phospholipid ratio determined. Suitable controls were included to correct for loss of material during the extraction procedure. As shown in Table III, small differences in the final sterol to phospholipid ratio occurred. The enhanced solubility of cholesterol and cholestanol had been reported previously[73]. These variations must be considered in the interpretation of the data.

Arrhenius curves of anisotropy of the various lecithin-sterol mixtures are shown in Figure V. In all cases addition of sterol

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TABLE III. STEROL TO PHOSPHOLIPID RATIO IN LIPSOMES CONTAINING VARIOUS STEROLS.

Sterol species	moles sterol / moles phospholipid
Cholestanol	0.31
Ergosterol	0.19
Stigmasterol	0.20
Cholesterol	0.27
Campesterol	0.23
Sitosterol	0.15
Epicholestanol	0.20
Cholestane	0.26
Zymosterol	not done
Ignosterol	not done

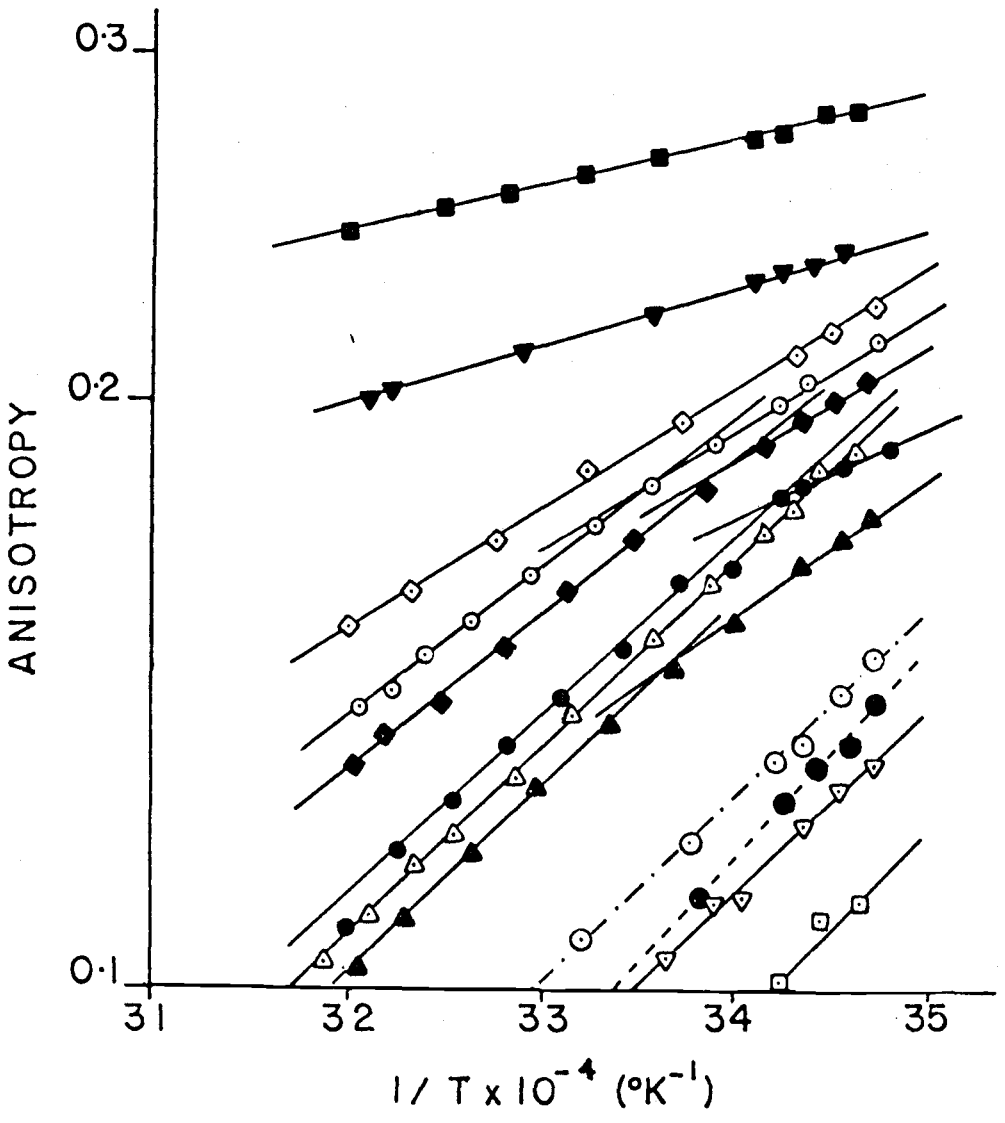

FIGURE VI. ARRHENIUS CURVES OF ANISOTROPY OF A FLUORESCENT PROBE EMBEDDED IN PHOSPHATIDYLCHOLINE BILAYERS. Liposomes were prepared and labelled with 1,6-diphenyl-1,3,5-hexatriene as described in Materials and Methods.

decreased the fluidity of the bilayer. The anisotropy values of the control, egg lecithin without sterol addition, are the lowest. It is apparent that qualitative changes in the sterol produce a wide range of fluidities, broader than the range seen in the mitochondrial preparations. The limited span of anisotropy values observed in the biological system suggest a cellular control mechanism to maintain fluidity within an optimal range for enzyme and membrane function. This will be discussed later.

Cholesterol, campesterol, stigmasterol, and sitosterol were selected to examine the effects of sterol side-chain modification on membrane fluidity. All have a β-hydroxyl at C-3 and an unsaturation at the five position in the B ring, differing only in unsaturation at C-22 and degree of alkylation at C-24. Cholesterol's saturated side chain without alkylation at C-24 was the most effective in decreasing the fluidity, i.e., ordering the bilayer. Fluidity was maintained by cholesterol within a very narrow range over the temperatures studied. Increasing the bulkiness of the substituent at C-24 with the addition of methyl or ethyl groups (campesterol and sitosterol) reduced the ability of the sterol to restrict the motion of the phospholipids in the membrane. The wide range of fluidity at the temperatures studied is reflected by the increased slope of the curves. Unsaturation at C-22 with an ethyl group at C-24 (stigmasterol) provided a more ordered bilayer than did sitosterol. This effect was anticipated as the introduction of an unsaturation in the sterol side chain produces a more rigid sterol molecule. The

increased rigidity hinders the motion of the phospholipid hydrocarbon chains. The observed results were predicted on the basis of molecular model building. While the rigid, planar sterol nucleus restricts the mobility of the portions of the bilayer proximal to the glycerol backbone, the motion of the distal portion may be increased by the introduction of an alkyl group at C-24. The unsubstituted sterol side chain does not appear sterically hindered and resembles the distal end of the fatty acyl chains. The effect of the sterol terminal isopropyl group is small as it interacts with only one or two of the fatty acyl carbons distal from the phospholipid glycerol backbone. Any disordering effect produced by the sterol chain in this region is not observed as this portion of the bilayer is already highly fluid. However, the rotation of the sterically hindered side chain produced by alkylation at C-24 interacts with a larger portion of the terminal hydrocarbon chain, thereby disrupting more extensively the interior of the bilayer.

These observations are in agreement with other reports[35,73,74,75]. Hsia *et al.*[74] and Butler *et al.*[75] showed that cholesterol exhibited the maximal effect in reducing the motion of the phospholipid hydrocarbon chains. Sitosterol was shown to be less effective.

To examine the effect of unsaturation in the nucleus, anisotropy data from the liposomes containing campesterol and ignosterol were compared. The dramatic increase in fluidity produced by ignosterol is reflected by the low anisotropy values. Whereas the nuclear ring structure of campesterol is planar, the conjugated unsaturation

at C-8 and C-14 pull the C and D rings of ignosterol out of the A-B plane. This is analagous to the comparison of Z008 and 8R1 in the biological system. In both cases, modification of the sterol structure to produce a less planar molecular increases the fluidity of the bilayer. Cholesterol and cholestanol, which differ only in unsaturation at C-5 may also be compared. Both molecules are planar, without alkyl substituents at C-24 and are expected to reduce significantly the fluidity of the membrane. As shown in Figure VI, incorporation of these sterol molecules into the bilayer does produce a less fluid membrane.

In the Arrhenius curves shown, an overall trend may be discerned which is related to the planarity of the sterol structure. Regardless of side chain unsaturations and alkylations, the planar sterol molecules (cholesterol, cholestanol, campesterol, stigmasterol, and sitosterol) produce a less fluid membrane than the nonplanar sterol ignosterol. Both ergosterol and zymosterol, whose deviation from planarity is intermediate between the extremes, exhibit an intermediate fluidity.

The significance of the phase transitions observed in the egg lecithin-sterol system is unclear. The phase transition of egg lecithin is well below the temperature range studied, and a discontinuity in the Arrhenius curve was not expected. The addition of sterol to a bilayer is generally associated with the elimination rather than the creation of a phase transition as demonstrated in differential scanning calorimetry studies. The presence of discontinuities only in the liposomes containing sterols from non-

mammalian systems suggests an incompatibility between the sterols and the phospholipid mixture derived from egg lecithin. The more planar molecules, which structurally resemble the mammalian sterol, cholesterol, appear to decrease the magnitude of the change in slope of the lines during the transition. Initially such a transition may arise from an inhomogeneous distribution of lipids in the bilayer, producing some highly ordered crystalline-like regions in an otherwise fluid bilayer. Evidence for the clustering of lipid species has been shown in artificial bilayer systems[27,79]. The incompatibility between mammalian phospholipid species and non-mammalian sterol is also suggested by the differences in fluidity of egg phosphatidylcholine and phospholipids extracted from S288c. Figure VI includes anisotropy measurements for the yeast phospholipids. The lowered anisotropy indicates that this mixture of phospholipids produces a more fluid bilayer than does the egg phosphatidylcholine.

The presence and configuration of the hydroxyl group at C-3 are also significant features of the sterol molecule. As shown in Figure V, addition of cholestanol to the bilayer provided a high degree of order, whereas its α-isomer, epicholestanol, had only a slight effect on bilayer fluidity. Cholestane was also ineffective in conferring order to the bilayer. Although important, the manner in which the hydroxyl group influences the interaction of the sterol with the bilayer is not clear. Some investigators have suggested the presence of hydrogen bonding between the phosphorous of

phosphatidylcholine and the sterol hydroxyl may be the underlying interaction orienting the sterol molecule in the bilayer[29,76,77]. Others find no direct interaction between the hydroxyl and any polar portion of the phosphatidylcholine molecule[35,78]. Furthermore, work with monolayers reported by Demel et al.[73] demonstrated that the configuration of the hydroxyl group does not affect the orientation of the sterol molecule at the air-water interface. Instead, hydration studies suggest that differences in hydration of the 3α-hydroxyl and the 3β-hydroxyl may be critical in the sterol-phospholipid interaction[72,80]. As shown by x-ray diffraction, the addition of water and cholesterol to a bilayer produces antagonistic effects. Cholesterol decreases the molecular area resulting in an increase in bilayer thickness. The thickening of the bilayer is an effect of enhanced membrane fluidity, the phase transition itself being associated with an increase in molecular area and a decrease in bilayer thickness[81,82]. The increased association of water with the bilayer increases the molecular area, thereby reducing the thickness of the membrane[80]. Therefore, differences in the hydration of the 3α- and 3β-hydroxyl affect the amount of water associated with the bilayer, indirectly altering membrane fluidity.

The data presented here indicate that the maximal orienting of the bilayer is produced on addition of a sterol molecule which is planar, has a β-hydroxyl at C-3 as well as a saturated, unalkylated side chain. This is consistent with the electron paramagnetic resonance and differential scanning calorimetric studies

on the effects of sterols on the mobility of the phospholipid fatty acyl chains[26,74,83,84]. The sterol derivatives cholesteryl methyl ether, 4-cholesten-3-one, thiocholesterol, and epicholesterol as well as sterols lacking the side chain at C-17 were ineffective in orienting the bilayer. Maximal decreases in fluidity were observed with lathosterol, 7-dehydrocholesterol and cholesterol whereas epicholestanol and β-sitosterol were unable to orient the bilayer[74,75]. Because the phase transition of egg phosphatidylcholine is below the temperature range studied only the orienting effects on incorporation of sterol in the bilayer were demonstrated. However, cholesterol, and presumably other sterols, function to fluidize bilayers in the gel phase by interrupting the close packing of the phospholipid fatty acyl chains[24,26,83,85]. This dual property suggests a buffering role for sterols in the membrane. As a bilayer buffer, sterol decreases the abruptness of the membrane transition, making fluid regions more ordered while also increasing the fluidity of ordered regions.

C. Response to Anisotonic Media

Yeast sphaeroplasts have been used by others to determine the effect of altering various membrane components, in particular sterols, on the ability of the plasma membrane to resist stretching[45]. The association of large amounts of sterol with the mitochondrial membranes[52] suggests that the mitochondria may also provide a system in which to study the role of sterols on membrane stretching capacity.

The response of MCC sphaeroplasts on dilution into anisotonic sucrose and sorbitol is shown in Figure VII. In both cases, the optical density of the suspension increases with increasing concentrations of osmotic stabilizer. However, the sugar alcohol sorbitol resulted in a higher optical density than did the sucrose. This may not reflect a difference in sphaeroplast size. Tedeschi and Harris[86] established a relationship between mitochondrial volume, optical density, wavelength of incident light, osmotic pressure and refractive index of the supportive medium. The relationship presumably could be extended to include the osmotic behavior of sphaeroplasts. The optical density measurement is dependent on the refractive index of the medium which in this case changes with the osmotic strength and hence accounts for the observed differences.

This dissimilar response to sucrose and sorbitol observed in sphaeroplasts prepared from different strains is shown in Figure VIII. No significant differences are observed between any of the organisms in either type of media. These data indicate that the ability of sphaeroplasts to resist stretching as determined by changes in optical density is not a function of sterol composition.

Mitochondria isolated from MCC responded to changing osmotic strength as shown in Figure IX. Again, the osmotic stabilizer used produced different effects. The optical density of mitochondria suspended in high concentrations of sorbitol was greater than the optical density in high concentrations of sucrose. At concentrations greater than 0.3 M sucrose, there was a decrease in abosrbance which

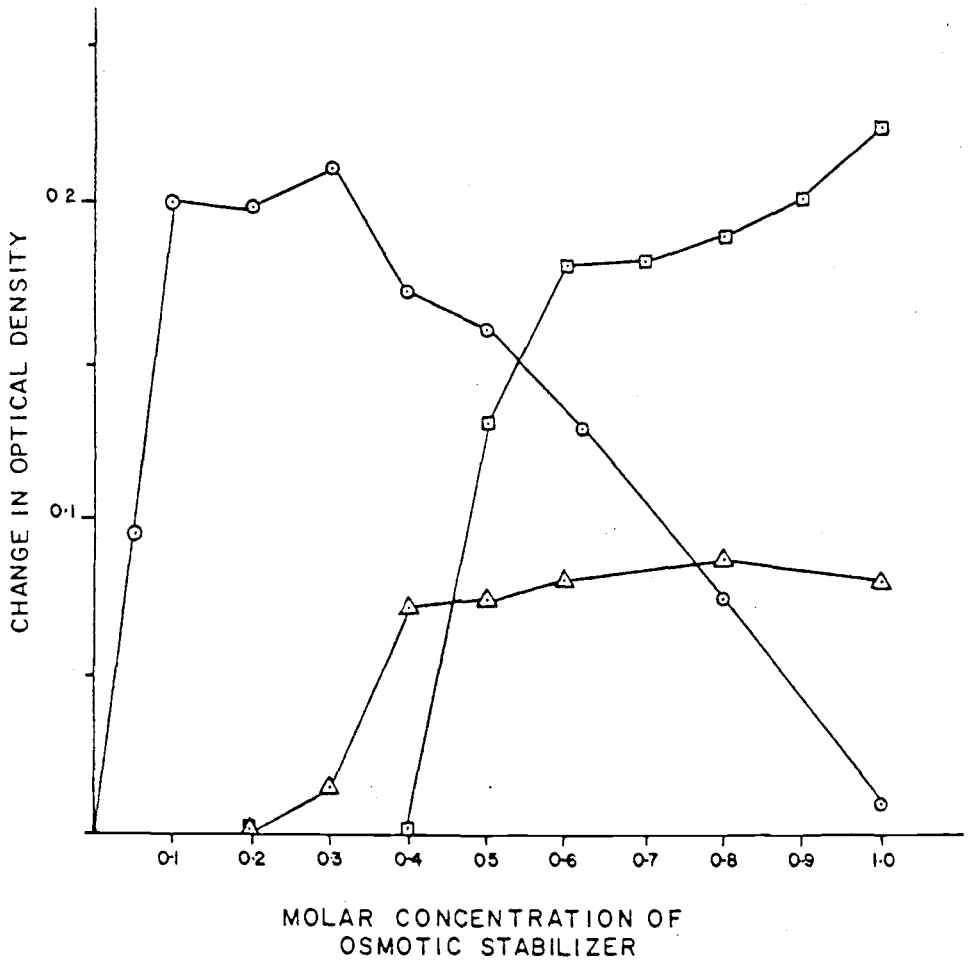

FIGURE VII. RESPONSE OF MCC SPHAEROPLASTS AND MITOCHONDRIA TO ANISOTONIC MEDIA. See materials and Methods for details of the experiment. ⊙--MCC mitochondria suspended in various concentrations of sorbitol; △-MCC sphaeroplasts resuspended in various concentrations of sucrose; □--MCC sphaeroplasts resuspended in various concentrations of sorbitol.

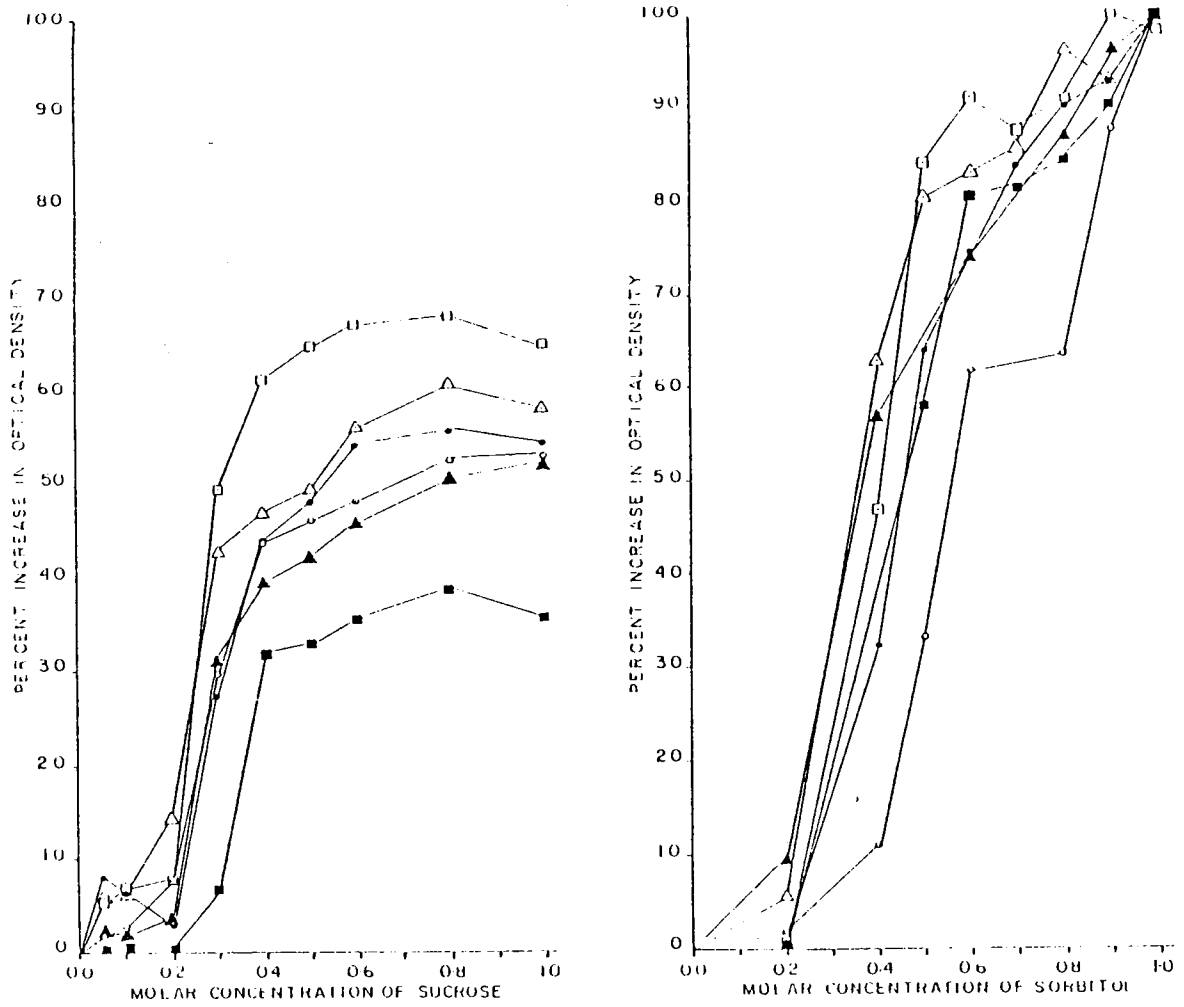

FIGURE VIII. RESPONSE OF SPHAEROPLASTS TO ANISOTONIC MEDIA. Figure A shows the response of sphaeroplasts prepared from the various strains to resuspension in a range of sucrose concentrations; B shows the response of the same preparations to resuspension in a range of sorbitol concentrations. The source of the sphaeroplasts is as follows: --MCC; --S288c; --3701b; --3701b-n3; --Z008; --8R1.

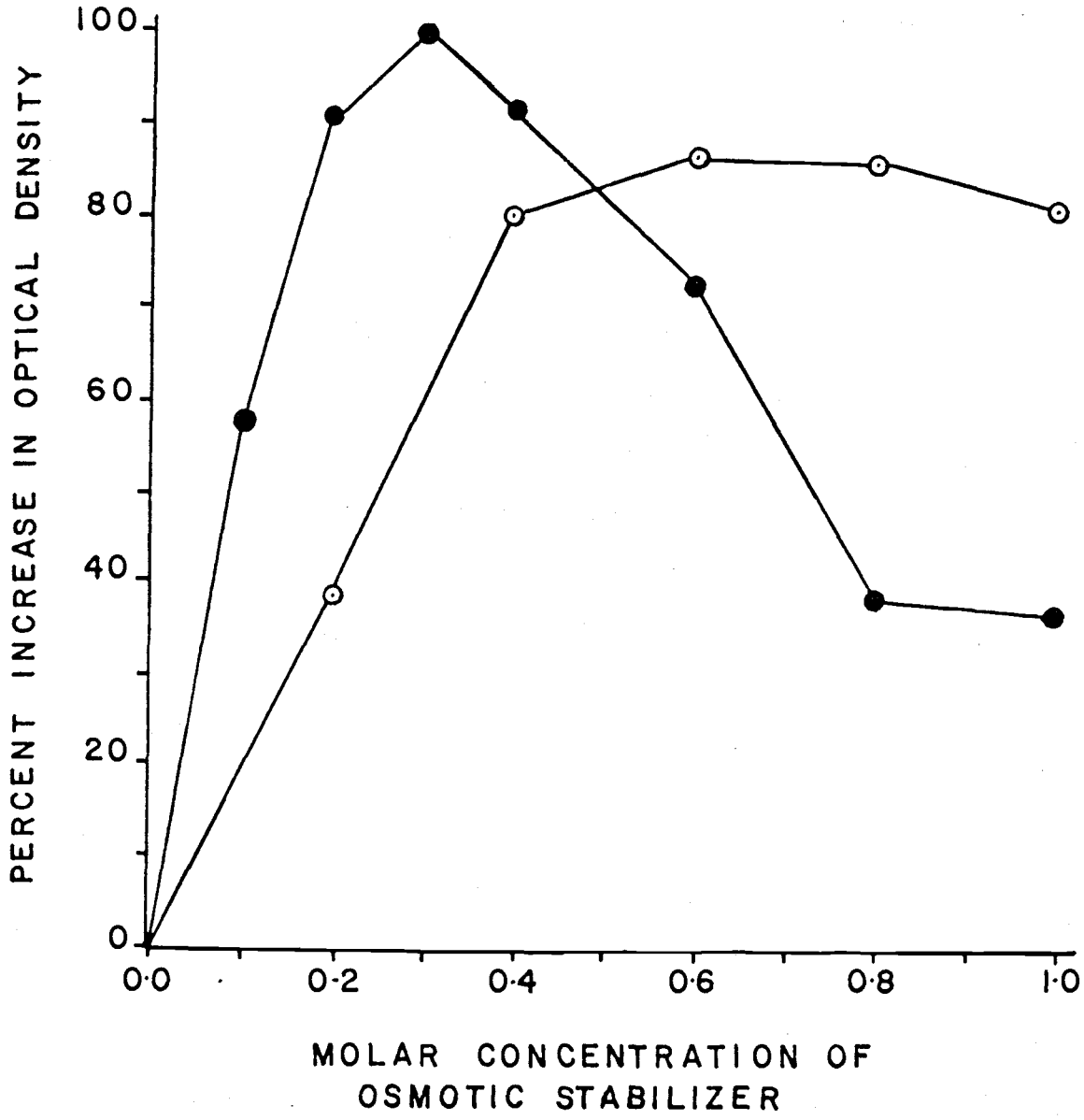

FIGURE IX. RESPONSE OF MCC MITOCHONDRIA TO ANISOTONIC MEDIA. Mitochondria were isolated and resuspended as described in Materials and Methods. ●--resuspension in various concentrations of sucrose; ⊙--resuspension in various concentrations of sorbitol.

was not observed with sorbitol. The response to sucrose is inconsistent with reports for rat liver mitochondria by Tapley[43] and Lehninger et al.[87] which showed increasing optical density with all increases in sucrose concentration.

It was originally thought that the high concentration of sucrose resulted in mitochondrial lysis, hence producing the low absorbance. The ability of the mitochondria to couple phosphorylation to respiration was selected as a criterion of intactness. Rates of respiration and coupled oxidative phosphorylation were measured in oxygraph buffer containing 0.9 M sorbitol, 0.6 M sorbitol, 0.2 M sorbitol, 0.2 M sucrose, 1.0 M sucrose or in the absence of osmotic stabilizers. The mitochondria used in this experiment were isolated from MCC. (See Table IV.) In the absence of osmotic stabilizer, mitochondria were unable to couple phosphorylation to respiration. High concentration of sorbitol permitted coupled respiration and produced the highest respiratory control ratio. The lower sorbitol concentration (0.2 M) was not effective in stabilizing the mitochondria. In this case, respiration was inhibited to a slight degree and no coupling was observed. Measurements made in the presence of 0.2 M and 1.0 M sucrose showed coupled respiration, although respiratory control is lower than that observed when sorbitol is used as the osmotic stabilizer. Previous reports indicate inhibition of respiration by high concentrations of sucrose[87]. The data reported here confirm this observation. It is not clear whether the lowered respiratory control results from a direct effect on enzyme activity or indirectly

TABLE IV. RESPIRATORY CONTROL AS A FUNCTION OF OSMOTIC STRENGTH. Respiratory rates were measured as described in Materials and Methods using 0.9 sorbitol, 0.6 M sorbitol, 0.2 M sorbitol, 0.2 M sucrose, 1.0 M sucrose and no osmotic stabilizer.

Stabilizer	Rate State 3	Rate State 4	Respiratory Control
0.9 M sorbitol	6.97×10^{-7}	1.36×10^{-6}	1.9
0.6 M sorbitol	6.97×10^{-7}	1.23×10^{-6}	1.7
0.2 M sorbitol	6.06×10^{-7}	-	-
0.0 M sorbitol	2.11×10^{-7}	-	-
0.2 M sucrose	8.98×10^{-7}	1.19×10^{-6}	1.3
1.0 M sucrose	6.51×10^{-7}	9.29×10^{-7}	1.4

from the inability of sucrose to fulfill the osmotic requirements necessary for coupled phosphorylation. The ability to couple phosphorylation to respiration where respiratory activity is inhibited as in the case of 1.0 M sucrose suggests the latter. The respiratory control exhibited in this condition, although lower than that observed with 1.0 M sorbitol, indicates mitochondrial intactness. Further experiments showed that the decrease in optical density at high concentrations of sucrose was reversible. Data are not shown. It therefore appeared that lysis was not causing the inconsistent optical behavior. However, the data were found to be in good agreement with Tedeschi and Harris[86] who observed a decrease in optical density at concentrations of sucrose above 0.3 molar. This decrease is attributed to variation of the refractive index of the supportive media.

On comparison of the behavior of mitochondria isolated from the different yeast strains, no differences were noted. (See Figure X) The data reported here using both enzymatically prepared sphaeroplasts and mitochondria are inconsistent with reports by Hossack and Rose.[45] Alterations in qualitative sterol composition do not appear to affect resistance to swelling. This has also been substantiated with artificial membrane studies. Demel et al.[35] reported no change in the osmotic behavior of egg lecithin vesicles containing different sterols.

D. L-Kynurenine-3-hydroxylase Activity

L-kynurenine-3-hydroxylase was selected for this study because of its location in the outer mitochondrial membrane. Thompson and Parks[19,88] have shown that a qualitative change in sterol composition alters the Arrhenius kinetics of cytochrome c oxidase and S-adenosyl methionine: $^{\Delta 24}$-sterol methyltransferase, both of which are located on the inner membrane. No data are available for enzymes located on the outer membrane. Such a study is of interest as the outer membrane has been reported to be sterol enriched[89,90]. Also, the phase changes observed in the fluorescence polarization study are much higher than those reported in the inner membrane enzymatic studies, suggesting that the fluorescent probe was embedded in the outer membrane.

Kynurenine hydroxylase catalyzes the addition of a hydroxyl group at the C-3 position of L-kynurenine. NADPH and molecular

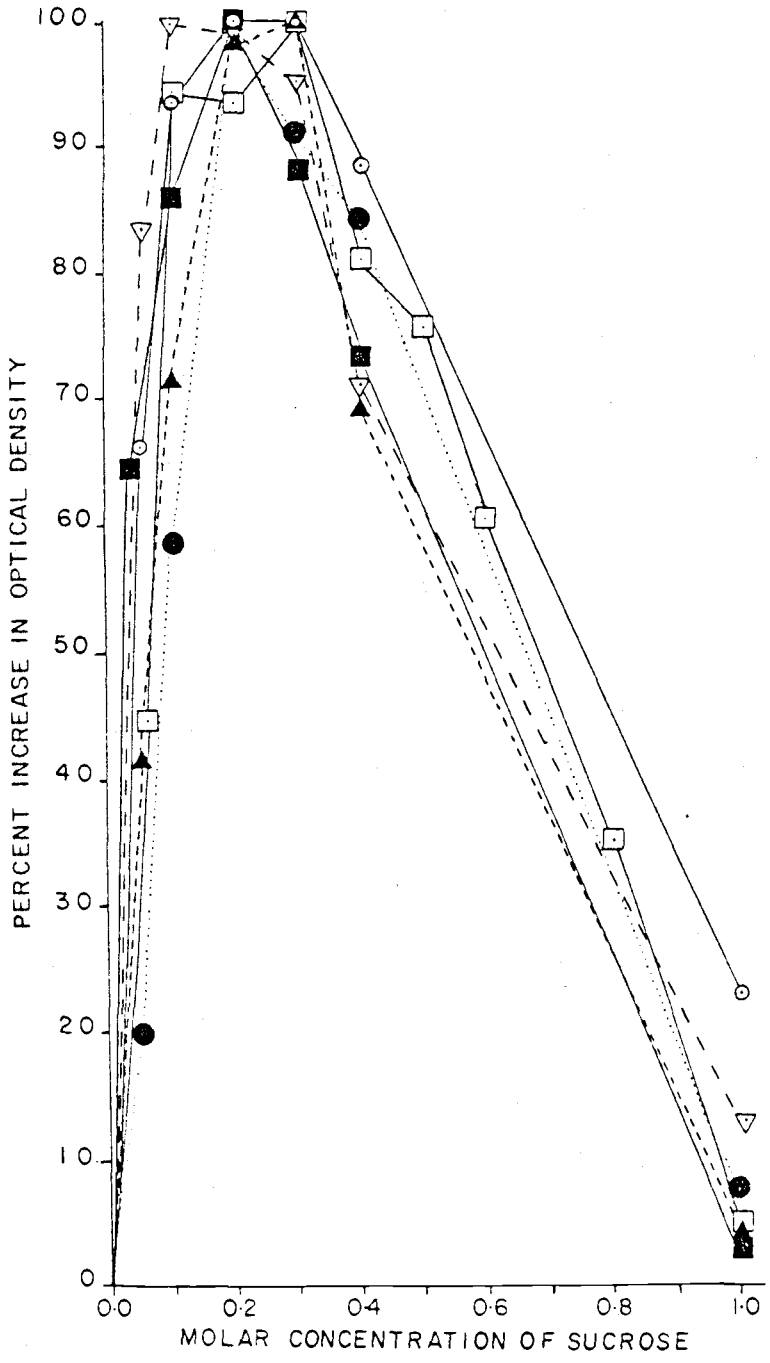

FIGURE X. RESPONSE OF MITOCHONDRIA TO VARIOUS CONCENTRATIONS OF SUCROSE. Details of the experiment are described in the text. The source of the mitochondria isolations are:-□---MCC; -▽--3701b; -■--3701b-n3; ●--Z008; ▲--8R1; ⊙--S288c.

oxygen are required. The rate is reported as the moles min^{-1} milligrams $protein^{-1}$ from the extinction coefficient reported by Linzen[91].

As shown in Figure XI the kynurenine hydroxylase preparations from the wild types have similar Arrhenius kinetics. The activation energies are 14.6 kilocalories and 14.2 kilocalories for S288c and 3701b, respectively. No discontinuities are observed in these two strains. However, MCC exhibits a discontinuity at 25.5°C. The activation energy of 16.0 kilocalories is lowered to 8.7 kilocalories above the transition. Although S288c and 3701b exhibit no discontinuity, no increase in activity in response to increased temperature is observed above 31 and 30.2°C, respectively, MCC activity leveling off in a similar manner at 34°C.

Arrhenius curves of enzyme activities in strains producing a sterol in addition to or in place of ergosterol showed increased activity as demonstrated by the displacement of the graphs. The activation energy for the enzyme preparation isolated from 3701b-n3 changed from 16.8 kilocalories to 9.6 kilocalories at 24.7°C. Identical changes in activation energy were observed in Z008 and 8RI at 24.8°Cand 22°C, respectively. In all cases, activity leveled off at 30°C.

A displacement of the Arrhenius curve of kynurenine hydroxylase activity appears to be characteristic of a qualitative sterol change. With the exception of MCC, the enzymatic and physical data are in agreement, indicating a sensitivity of the kynurenine hydroxylase

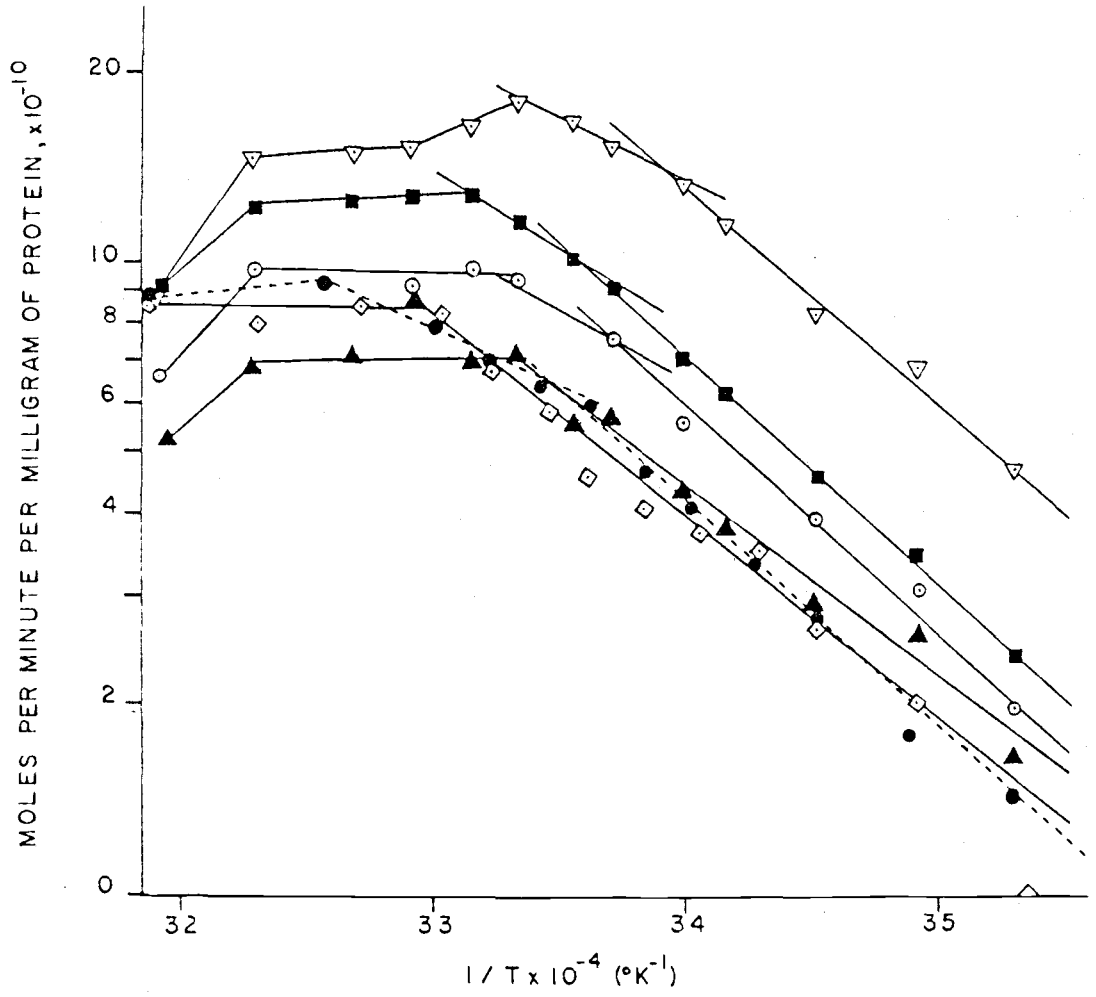

FIGURE XI. ARRHENIUS CURVES OF KYNURENINE HYDROXYLASE ACTIVITY. See text for details. The mitochondrial preparations are labelled as follows: ●--MCC; ▲--3701b-n3; --Z008; --8R1; --S288c.

activity to the phase of the membrane. The inconsistency seen between the physical and enzymatic data in the MCC mitochondrial preparations may reflect the high sterol to phospholipid ratio of this strain, the kynurenine hydroxylase being partitioned into a less fluid region of the membrane. The levelling off of enzyme activity observed in all preparations between 30 and 38°C results from an irreversible thermal inactivation of the enzyme. The data are not shown. The values reported at temperatures above 30°C are not true rates, but an artifact of the way the rate was determined.

E. $MTPP^+$ Uptake

Growth studies by Gonzales[92] demonstrated that 3701b-n3 was incapable of growth on respiratory substrates at temperatures above 30°C, whereas growth of the parental was unaffected until the temperature reached 38°C. This suggested that the mitochondria were defective in energy production and in light of Haslam's report[42], suggested that the permeability of the mitochondria might be altered by the qualitative sterol change. Direct measurement of passive proton permeability was largely unsuccessful. However, the uptake of the lipophilic cation methyltriphenylphosphonium ($MTPP^+$) offered another means of measuring membrane permeability. This cation has been used for the determination of the transmembrane electrical potential ($\Delta\psi$) in Escherichia coli membrane vesicles[93,94] and SV40 transformed mouse fibroblasts[95]. The ions are sufficiently lipophilic to pass through a lipid bilayer on generation of a transmembrane electrical potential where there is a net negative charge

inside the vesicle. The method is attractive in that the transmembrane electrical potential generated on addition of a suitable respiratory substrate can be measured.

As shown in Figure XII the addition of ethanol as a respiratory substrate resulted in the uptake of $MTPP^+$. When either of the uncouplers 2,4-dinitrophenol or carbonyl cyanide-m-chlorophenylhydrazone was added, a reduced amount of the ion was accumulated. Note that even in the absence of a respiratory substrate $MTPP^+$ was accumulated, indicating maintenance of a membrane potential. The addition of 2,4-dinitrophenol or carbonyl cyanide-m-chlorophenyhdrazone was required to eliminate this accumulation.

The uptake of probe responds to changes in the potential. The sample marked with the open circles was preincubated in the presence of respiratory substrate and labelled $MTPP^+$. At T=0, carbonyl cyanide-m-chlorophenylhydrazone was added. The rapid efflux of label demonstrated that the constant level of probe observed in the other experiments represented a steady state condition rather than the conversion of the label to a stable mitochondrial product. Addition of the respiratory inhibitor KCN was ineffective in abolishing the gradient. A similar effect was noted by Shuldiner and Kaback[93] in a study of Escherichia coli vesicles and appears to be related to the portion of the respiratory chain where inhibition occurs.

A number of respiratory substrates stimulated uptake of $MTPP^+$. (See Table V.) The ability to stimulate cation uptake corresponded

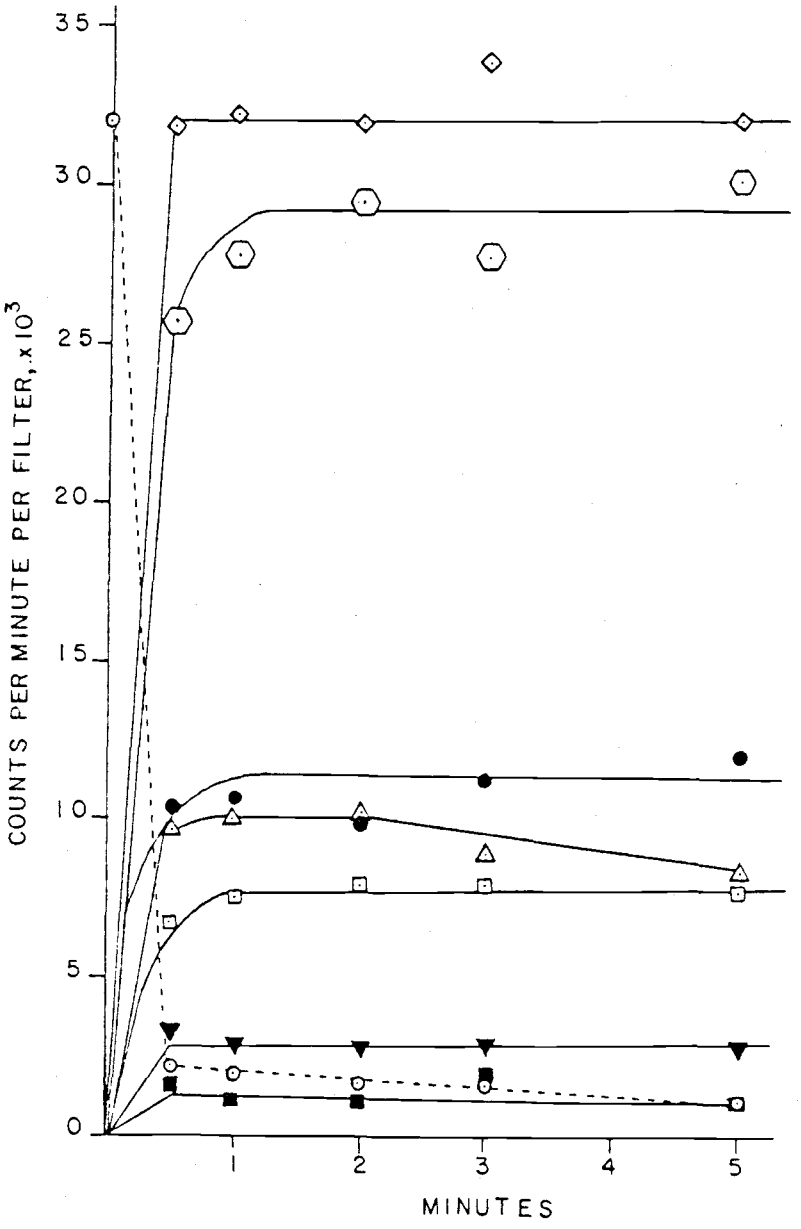

FIGURE XII. UPTAKE OF $MTPP^+$ IN THE PRESENCE OF UNCOUPLERS. Fifty microliters of a mitochondrial suspension isolated from MCC were added to the reaction buffer as described in Materials and Methods. The following additions were made to the reaction mixture prior to the preincubation period: ◇--two percent ethanol; ⊙--two percent ethanol, 5×10^{-5} molar KCN; ●--two percent ethanol, 5×10^{-5} molar 2,4-dinitrophenol; △--no addition; ⊡--two percent ethanol, 1×10^{-5} molar carbonyl cyanide-m-chlorophenylhydrazone; ▼--5×10^{-5} molar 2,4-dinitrophenol; ■--1×10^{-5} molar carbonyl cyanide-m-chlorophenylhydrazone. The open circles, ⊙, represent the addition of carbonyl cyanide-m-chlorophenylhydrazone at T = 0 following a two minute preincubation in the presence of two percent ethanol and $MTPP^+$. See text for details.

with the ability to serve as a respiratory substrate for oxidative phosphorylation. All substrates tested stimulated oxygen uptake. However, oxygen consumption supported by glycerol did not respond to the uncoupler carbonyl cyanide-m-chlorophenylhydrazone, indicating a non-respiratory oxygenase activity. All substrates except glycerol also stimulated accumulation of $MTPP^+$. It is not clear why α-ketoglutarate, succinate, and pyruvate failed to show respiratory control. Coupling of phosphorylation to respiration when other respiratory substrates were used indicates functional integrity of the mitochondrion.

As shown in Figure XIII, the steady state level of $MTPP^+$ was linear with respect to the amount of protein in the reaction mixture. In addition, the steady state level was a linear function of $MTPP^+$ concentration from two to 26 micromolar as shown in Figure XIV. These data are in accord with the concept of a passive equilibration of the probe in response to a potential difference across the membrane.

Because accumulation responds to the membrane potential and is a non-carrier mediated process the electrical potential may be calculated from the Nernst equation. At the steady state:

$$\Delta\psi = -\frac{RT}{F} \ln \frac{[MTPP^+]_{in}}{[MTPP^+]_{out}}$$

The apparent accumulation of $MTPP^+$ varied significantly between replica measurements and necessitated the determination of $\Delta\psi$ from a large number of samples. Each calculation represents the average of

TABLE V. STIMULATION OF $MTPP^+$ UPTAKE BY RESPIRATORY SUBSTRATES. Oxygen of a mitochondrial preparation isolated from MCC was measured as described in Materials and Methods using various respiratory substrates. Pyruvate, glycerol, and lactate were added to 0.5 percent. All other substrates were added to give a final concentration of 6.25×10^{-3} molar. CCCP = carbonyl cyanide-m-chlorophenylhydrazone.

Substrate	$\frac{\text{moles}}{\text{min mg protein}}$	Respiratory Control	Response to CCCP	CPM ^{14}C-$MTPP^+$ 0.01 mg protein
ethanol	2.8×10^{-7}	1.56	yes	14198 ± 1176
α-ketoglutarate	1.2×10^{-7}	-	yes	14420 ± 2031
succinate	1.8×10^{-7}	-	yes	14453 ± 1373
glycerol-3-phosphate	4.2×10^{-7}	1.44	yes	14873 ± 2090
citrate	7.6×10^{-8}	1.47	yes	14452 ± 1373
NADPH	2.4×10^{-7}	1.2	yes	13847 ± 2047
pyruvate	1.0×10^{-7}	-	yes	13834 ± 1907
glycerol	5.9×10^{-8}	-	no	7808 ± 629
lactate	2.9×10^{-7}	1.2	yes	14443 ± 1692

30 determinations. In all cases, the standard deviation is less than ten percent. Uptake of $MTPP^+$ was monitored at 17°C, 27°C, and 35°C to determine the relationship between qualitative sterol changes and the ability to establish and maintain a transmembrane potential over a broad temperature range.

As shown in Table VI, little variation is observed in the calculated potentials for the mitochondria prepared from MCC, S288c, 3701b, and 3701b-n3. The potential for 3701b-n3 is higher than the potential

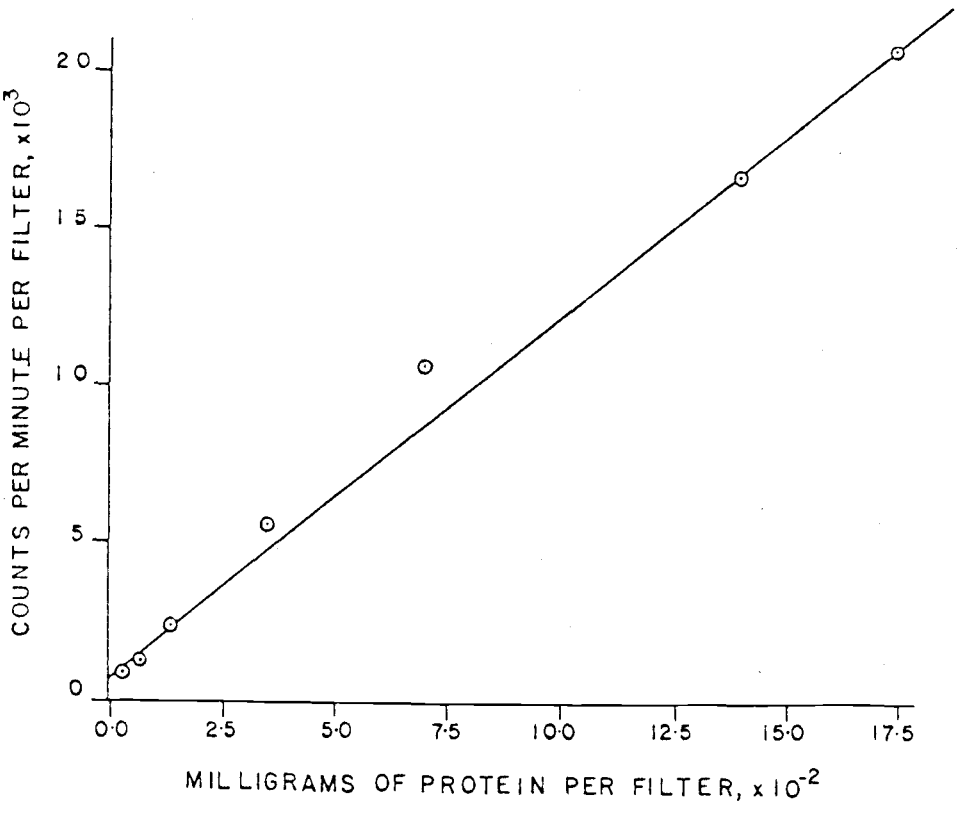

FIGURE XIII. UPTAKE OF $MTPP^+$ WITH RESPECT TO CONCENTRATION OF PROTEIN. Uptake of $MTPP^+$ by mitochondria isolated from S288c over a range of protein concentrations was determined as described in Materials and Methods.

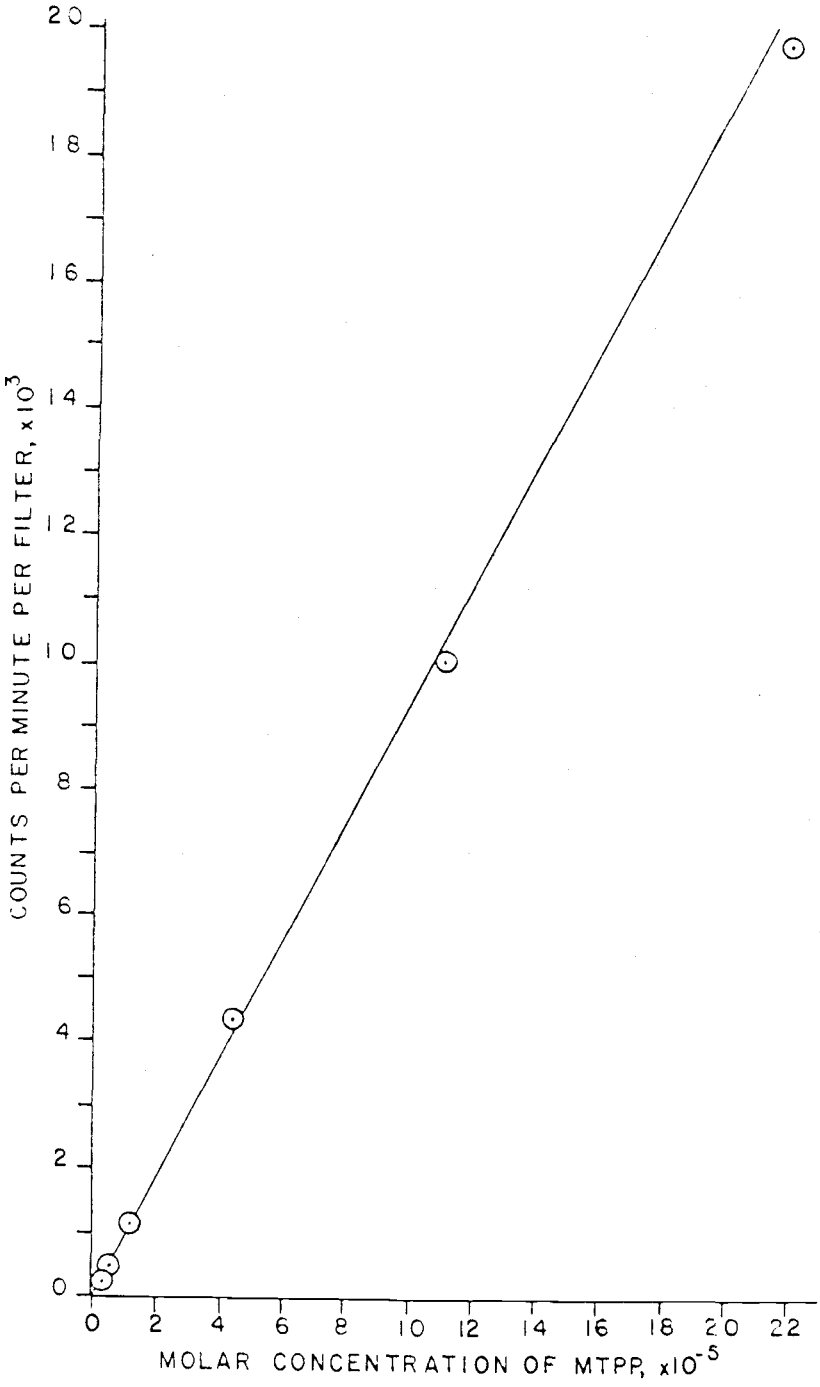

FIGURE XIV. UPTAKE OF THE LIPOPHILIC PROBE AS A FUNCTION OF $MTPP^+$ CONCENTRATION. Uptake of $MTPP^+$ by mitochondria isolated from MCC was determined as described in Materials and Methods over the concentration range two to 26 micromolar.

TABLE VI. TRANSMEMBRANE POTENTIAL AND RESPIRATORY COMPETENCE OF YEAST STRAINS. The potential was calculated from the uptake of the lipophilic cation ^{14}C-MTPP$^+$ as described in Materials and Methods. The percent respiratory control is calculated as the product of the respiratory control ratio at 17°C or 35°C and the reciprocal of the respiratory control ratio at 27°C.

Strain	Temperature	$\Delta\psi$	Percent Respiratory Control
MCC	17	160	80.7
	27	136	100
	35	133	61.8
3701b	17	138	87.9
	27	140	100
	35	127	92.5
3701b-n3	17	145	89.7
	27	147	100
	35	139	79
Z008	17	115	78.5
	27	120	100
	35	112	78.5
8R1	17	119	64.0
	27	125	100
	35	117	86.2
S288c	17	146	87.2
	27	133	100
	37	121	58.8

for 3701b at all temperatures. Both Z008 and 8R1 have a potential ten millivolts lower than the other organisms, suggesting a relationship between the sterol side chain and the maintenance of the potential. However, since no significant difference is observed between the two strains, this dissimilarity may be attributed simply to strain variation. There appears to be no correction between sterol structure and the ability to maintain a transmembrane potential.

This was also reflected in the ability of the mitochondria isolated from the various strains to couple phosphorylation to respiration. All of the $MTPP^+$ uptake experiments were conducted with respiratory competent mitochondria. The maximum respiratory control in all cases was observed at 27°C, it being diminished both at 17°C and 35°C. However, there was no significant difference in loss of respiratory competency between the strains examined, i.e., the diminution with temperature was proportional in all cases. Similar observations were made in Hough's study[96] of respiratory control in 3701b and 3701b-n3. Furthermore, the data suggests that there is some other defect caused by sterol alteration which inhibits the growth of 3701b-n3 on respiratory substrates at temperatures above 30°C. A recent isolation of sterol mutants from 3701b and S288c by Jensen et al.[100] showed a large number of the 3701b mutant clones were incapable of growth on respiratory substrates above 30°C, whereas mutants from S288c showing the same sterol phenotype were capable of growth, suggesting that the 3701b-n3 growth profile is a characteristic of the parental strain rather than a direct effect of sterol alteration on mitochondrial energy production.

The fluorescence polarization data suggested that the fluidity of the mitochondrial membrane was restricted to a narrow range of possible fluidities, even when sterols other than ergosterol were incorporated in the membrane. The observation that qualitative sterol changes do not alter the transmembrane potential supports this hypothesis. Since there is no significant change in the degree of unsaturation of the phospholipid fatty acyl chains or in the sterol

to phospholipid ratio in the isogenic strains, this control may be exerted by manipulation of the phospholipid head group composition. The maintenance of a functional membrane was suggested by Schatz[1,2]. Under conditions of anaerobiosis where unsaturated fatty acids cannot be synthesized, short chained fatty acids are the dominant species produced. Decreasing the chain length of the phospholipids produces the same effect as the introduction of unsaturations - an increase in bilayer fluidity. When oxygen is not available for the biosynthesis of unsaturated fatty acids, the controlling mechanism compensates by decreasing the fatty acid chain length. The mutant selections of Karst et al.[97], Trocha et al.[98] and Bard et al.[99] indicate that a wide variety of sterols can be accomodated in the membrane of Saccharomyces cerevisiae. Mutants representing blocks at most steps in the sterol biosynthetic pathway have been isolated, indicating that these are not lethal mutations. However, the ability appears to be strain dependent. Mutant isolation by Jensen et al.[100] shows that certain wild type parentals are capable of producing distinct classes of sterol mutants. Some strains produce a variety of sterol classes whereas others produce only one. Strain S288c produces nine different sterol classes whereas 3701b produces only the 3701b-n3 sterol phenotype. This may reflect the inability of 3701b to vary its membrane properties to accomodate sterols other than ergosterol and its Δ7,22-derivative. As shown above, the cellular sterol to phospholipid ratio of S288c varies dramatically during the growth phase, whereas the change in 3701b was much smaller, indicating a potential ability of S288c to alter its membrane properties. The ability to control fluidity varies from strain to strain.

V. Summary

When sterols other than ergosterol are incorporated into the mitochondrial membrane of Saccharomyces cerevisiae, Arrhenius curves of the mobility of a fluorescent probe embedded in the bilayer exhibit discontinuities, apparently reflecting a change in the phase of the membrane. The effect of qualitative alteration of sterol composition on membrane fluidity was demonstrated in a parallel study in which various sterols were incorporated into artificial phosphatidylcholine bilayers. The maximal orienting effect occurred when planar sterol molecules, possessing a 3β-hydroxyl without an unsaturated or alkylated side chain were incorporated into the bilayer. Non-planar sterol molecules were not as effective in ordering the membrane. These data are consistent with previously reported electron paramagnetic resonance studies of artificial systems. In the biological study, the fluidity changes seen on comparison of the wild-types with the isogenic mutants which accumulate sterols other than ergosterol are consistent with the predictions made from the artificial membrane study and molecular model building; mutations resulting in the production of a more planar sterol molecule contributed to a more ordered membrane. No change in the fatty acid composition of the phospholipids or the sterol to phospholipid ratio was seen in the isogenic strains. Differences in fluidity were observed in the non-isogenic wild types. Two of these strains, 3701b and S288c, exhibited different sterol to phospholipid ratios but virtually identical membrane fluidity, suggesting

a role for phospholipid head groups in the maintenance of membrane fluidity.

Arrhenius kinetics of L-kynurenine-3-hydroxylase activity, an enzyme embedded in the outer mitochondrial membrane, also exhibited discontinuities. These transitions occurred within a degree of those measured in the fluorescence polarization study, indicating the sensitivity of this enzyme to the bulk phase of the membrane.

Although qualitative sterol alterations are capable of affecting mitochondrial membrane fluidity as monitored by the mobility of a flourescent probe and the activity of a membrane bound enzyme, no apparent effect on the conversion of energy into a usable chemical form by the mitochondria was observed. The mitochondrial preparations were all capable of generating a transmembrane potential and coupling phosphorylation to respiration at the three temperatures examined. These data, along with the observation of preferential deposition of ergosterol and phospholipids rich in 16:1 fatty acyl chains in the mitochondrial membrane suggests that mitochondrial functionality may be conserved when qualitative sterol alteration occurs by redistribution of phospholipid species. This is currently under investigation.

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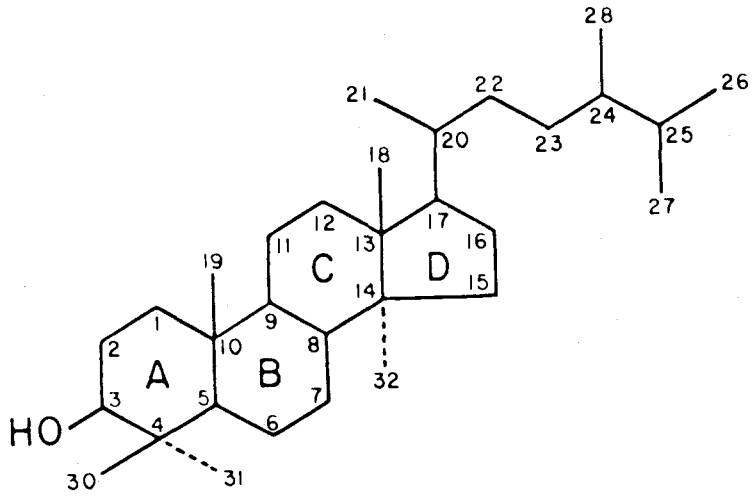
APPENDIX



STEROL NUMBERING SYSTEM. The sterol numbering system is shown for a saturated 31 carbon methyl lanosterol. Each carbon is assigned a number. Sterols of the 28 carbon series, such as ergosterol, lack carbons 30, 31, and 32. Cholesterol derivatives lack carbons 28, 30, 31, and 32. Carbon 29, not shown here, designates an ethyl group at the C-24 position. The position of unsaturations are indicated by using the whole number of the first carbon of the bond; the second carbon is assumed to be the succeeding number of the sequence. Where this is not the case, the number is followed by a second number in parentheses to indicate that the double bond is to a carbon other than the next sequential number.