1977

The Effect of Ergosterol on the Desaturation Of 14C-Labeled Oleic Acid in Tetrahymena

Maynard E. Neville Jr.
Eastern Illinois University

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THE EFFECT OF ERGOSTEROL ON THE DESATURATION OF ^{14}C-LABELED OLEIC ACID IN TETRAHYMENA

(TITLE)

BY

MAYNARD E. NEVILLE JR.

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE IN ZOOLOGY

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1977
YEAR

I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
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THE EFFECT OF ERGOSTEROL ON THE DESATURATION OF ^{14}C-LABELED OLEIC ACID IN *TETRAHYMENA*

BY

MAYNARD E. NEVILLE JR.

B. S. in Zoology, Eastern Illinois University, 1976

ABSTRACT OF A THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Science in Zoology at the Graduate School of Eastern Illinois University

CHARLESTON, ILLINOIS
1977

The ciliated protozoan Tetrahymena pyriformis-Strain W desaturated ^{14}C-labeled fatty acid substrate in both sterol supplemented (ergosterol) and nonsupplemented cultures. Cis-octadecenoic acid (^{14}C-9-18:1), when added to the medium during the log phase of growth, was incorporated into the cells and also desaturated at the Δ^6 and Δ^{12} positions. The ^{14}C labeled substrate and products, linoleate (9,12-18:2) or linolenate (6,9,12-18:3) were recovered and separated by silver-nitrate Unisil column chromatography. Initially, recovery of ^{14}C compounds varied widely when expressed as the percent of total counts per minute recovered from column chromotography, and a variety of procedural modifications were required before a reproducibly significant effect of the foreign sterol on desaturation of substrate was demonstrated.

Addition of ergosterol to the growth medium of Tetrahymena leads to incorporation of the foreign sterol within cell membranes and supression of synthesis of the native compound tetrahymanol, as well as to changes in the fatty acid compositions of several major classes of membrane lipid. Alteration of fatty acid composition is thought to represent a regulatory mechanism whereby optimum membrane fluidity is maintained when

the slightly dissimilar foreign sterol is incorporated into the phospholipid bilayer of the membranes.

The present study, using several different conditions of growth temperature, culture size and ergosterol concentrations, is an attempt to provide evidence supporting a regulatory role for membrane-bound sterol. The regulatory mechanism hypothesized is allosteric modification of an enzyme or enzymes involved in the synthesis of fatty acids contained in membrane phospholipids by the membrane-bound sterol. Such a mechanism could account, at least in part, for the balance between sterol and fatty acid content of membranes. The data presented here show that a statistically significant decrease in desaturation of ^{14}C-labeled 9-18:1 can be demonstrated in Tetrahymena cell cultures whose membranes contain the foreign sterol ergosterol when compared to cell cultures not receiving sterol supplementation when growth temperature is maintained at 20°.

Acknowledgements

I wish to thank Dr. Karen Ferguson for her generous support and encouragement during the course of my work at E. I. U. It has been a unique and rewarding experience, and one for whioh I am very grateful.

A special thanks to my wife Pam, for trying so hard to understand.

Eastern Illinois University

THE EFFECT OF ERGOSTEROL ON THE DESATURATION OF ^{14}C-LABELED OLEIC ACID IN *TETRAHYMENA*

by

Maynard E. Neville Jr.

Presented to the Faculty of Eastern Illinois University
in Partial Fulfillment of the Requirements for
the Degree of Master of Science

Charleston, Illinois
December, 1977

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Terms and Abbreviations

1. fatty acid nomenclature
   a. a,b-X:0 = position of double bonds -- carbon chain length: number of double bonds.
      Example: 9,12-18:2; double bonds at carbons 9 and 12 in an 18 carbon chain: 2 double bonds
   b. oleate -- 9-18:1 (oleic acid) (octadecenoic acid)
      linoleate -- 9,12-18:2
      linolenate -- 6,9,12-18:3
   c. Δ^6 -- refers to the position of the double bond in the chain.
2. boundary lipid -- distinguishes lipid associated with protein from other lipid of the membrane bilayer (bulk lipid); frequently termed annular lipid (annulus) when refering to lipid associated with enzymes.
3. CM2:1 -- chloroform methanol 2:1 v:v
4. desaturase -- an enzyme which inserts a double bond in the fatty acid chain; i.e. 6-desaturase inserts a double bond at the 6th carbon from the carboxyl group.
5. desaturation index -- summation of (double bonds in each fatty acid) X (mol % of each fatty acid)

Summary

The ciliated protozoan Tetrahymena pyriformis-Strain W desaturated ^{14}C-labeled fatty acid substrate in both sterol supplemented (ergosterol) and nonsupplemented cultures. Cis-octadecenoic acid (^{14}C-9-18:1), when added to the medium during the log phase of growth, was incorporated into the cells and also desaturated at the Δ^6 and Δ^{12} positions. The ^{14}C labeled substrate and products, linoleate (9,12-18:2) or linolenate (6,9,12-18:3) were recovered and separated by silver-nitrate Unisil column chromatography. Initially, recovery of ^{14}C compounds varied widely when expressed as the percent of total counts per minute recovered from column chromotography and a variety of procedural modifications were required before a reproducibly significant effect of the foreign sterol on desaturation of substrate was demonstrated.

Addition of ergosterol to the growth medium of Tetrahymena leads to incorporation of the foreign sterol within cell membranes and supression of synthesis of the native compound tetrahymanol (1), as well as to changes in the fatty acid compositions of several major classes of membrane lipid (2). Alteration of fatty acid composition is thought to represent a regulatory mechanism

whereby optimum membrane fluidity is maintained when the slightly dissimilar foreign sterol is incorporated into the phospholipid bilayer of the membranes.

The present study, using several different conditions of growth temperature, culture size and ergosterol concentrations, is an attempt to provide evidence supporting a regulatory role for membrane-bound sterol. The regulatory mechanism hypothesized is allosteric modification of an enzyme or enzymes involved in the synthesis of fatty acids contained in membrane phospholipids by the membrane-bound sterol. Such a mechanism could account, at least in part, for the balance between sterol and fatty acid content of membranes. The data presented here show that a statistically significant decrease in desaturation of ^{14}C-labeled 9-18:1 can be demonstrated in Tetrahymena cell cultures whose membranes contain the foreign sterol ergosterol when compared to cell cultures not receiving sterol supplementation when growth temperature is maintained at 20°.

Introduction

As the investigation of metabolism at the cellular level continues, the internal and limiting membranes of cells increasingly are being characterized as dynamic organelles contributing to a variety of functions (3). Transport, receptor, synthetic and regulatory roles are becoming established. Further, a large amount of raw material required by higher organisms originates in the photosynthetic process carried out at the chloroplast membrane in green plants. Systems based within or on membranes are essential to organization and effective interaction within the cellular environment. Recent elucidation of the role of membrane insulin receptors (4) and receptor control of cholesterol metabolism (5) are current examples. The membrane increasingly must be characterized as an active chemical participant as well as a physical presence.

The unit membrane hypothesis was advanced by Robertson (6) and was the basis for the currently accepted Fluid Mosaic Model proposed by Singer (7). While retaining many of the previous basic concepts, this latter, more dynamic model allows for a variety of molecular components and recognizes a variable thickness and molecular composition of membranes according to their cellular location. Basically it visualizes a hydrophobic barrier to the aqueous environment created by a lipid bilayer approximately 45Å thick.

This bilayer is patterned after the micelles formed spontaneously by representative amphipathic molecules (8). Most of the lipid bilayer is contributed by the phospholipids of the cell, which are structurally capable of participating in the polar and nonpolar relations required. Protein molecules are postulated to associate with the lipid bilayer to varying extents (integral (7), or peripheral (7,9,10,11)) depending on the degree of their hydrophobic character. Most proteins are pictured in a globular conformation and as containing additional functional groups such as carbohydrate or lipid groups. It should be emphasized that in relation to the relative abundance of information concerning soluble proteins, little is known about membrane-bound structural proteins and enzymes.

Membrane lipids fall into four general categories: glycerophospholipids (I), sterols (II), carotenoids (III), and sphingolipids (IV)(Figure I). Along with the usual complement of these lipids, Tetrahymena membranes contain phosphonolipids in which carbon is bonded directly to phosphorous (see X = $-CH_2CH_2NH_3^+$ in Figure I).

Addition of the fatty acids to the lipid backbone takes place in a series of steps beginning with activation of the fatty acid to the CoA derivative by an

energy-requiring conversion. In this form it is elongated and/or desaturated to the required structure. Insertion into phospholipid is aocomplished by an aoyl-transferase enzyme.

Sterol molecules are bulky, multi-ring amphipathic molecules and are major components of eucaryotic cytoplasmic membranes at molar ratios of sterol to phospholipid of 0.5 to 0.8. The structure of the native sterol-like triterpene alcohol found in Tetrahymena (tetrahymanol) is only slightly different from that of a true sterol, ergosterol. Figure I illustrates the structure of the two molecules.

Interaction between the lipid and protein components of membranes has been investigated by a variety of methods, including electron spin resonance (ESR)(12), electron microscopy (13), X-Ray diffraction (14) and reconstitution (15). G. B. Warren et. al. (15) showed that sarooplasmic reticulum Ca^{++}- ATPase had an absolute requirement for a lipid environment to maintain activity and that this environment consists of a minimum of 30 phospholipid molecules per ATPase molecule. This same work demonstrated a preference with respect to activity of the enzyme molecule for the presence of certain acyl side chains in this boundary lipid, discriminating

according to chain length and degree of uneaturation. Cholesterol was shown to be excluded from the lipid annulus.

An effect of protein on lipid has also been demonstrated. Using spin label technique (16) it was shown that integral proteins prevent phase transitions in the boundary (annular) lipid. This pattern of alteration is, however, confined to the annulus and is not seen in the bulk lipid. This observation supports the concept of a special protein-lipid domain within the bilayer. Again, exclusion of cholesterol from this (enzyme) annulus was indicated.

In addition to these influences of lipid on protein and protein on lipid, consideration has been given to the interaction between sterol and lipid as an explication of the Fluid Mosaic Model. Rothman and Engleman (17) have proposed a model containing several principles outlining the energetic preference of cholesterol for bonding to phospholipid hydrocarbon side chains, as opposed to cholesterol-cholesterol interactions. Cholesterol is sometimes referred to as the 'universal equalizer' for its role in influencing the physical properties of phospholipid (18). The presence of saturated fatty acyl groups on the glycerol backbone

naturally causes a phospholipid to tend toward the solid or gelled state while unsaturated groups make it more fluid (19). Intermediate between these two states is a mixed phase that allows for lateral movement of proteins, required for their function, through the bilayer. From calorimetry (20) there is known to be a tendency for phospholipids containing a mixture of acyl side chains to show a wide temperature range through which the mixed phase condition is maintained, while the phase transition of a pure lipid is fairly sharp. The presence of a sterol so widens the mixed phase temperature range that the delineation of the upper (T_h) and lower (T_l) temperature boundaries are obsoured. It has been shown using spin label techniques (21) that the presence of a cis double bond in the fatty acyl side chain disrupts the association (packing order) of the side chains relative to that exhibited by saturated acyl groups. It is speculated that the sterol molecule acts as a unifying bridge between unsaturated aoyl groups of adjacent phospholipid molecules exerting a stabilizing effect. Conversely, the attraction between adjacent long straight chain hydrocarbon groups, favoring the gelled state, is disrupted by insertion of the sterol. Thus the sterol physically contributes to the membrane's

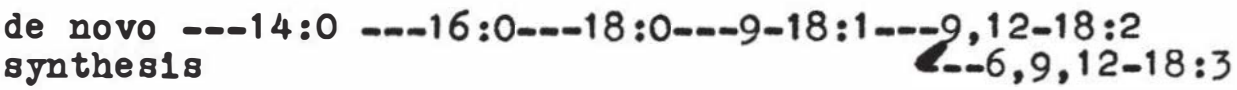
ability to resist formation of lipid microenvironments, that is, localized regions (all gelled or too fluid) which might disrupt membrane function by contributing to a suboptimum environment for protein activity and/or decreasing bilayer structural integrity (20). Such structural influences have been demonstrated in Mycoplasma cells and liposomes (22), whose permeability to nonelectrolytes is quite dependent on the structure and chainlength of the phospholipid fatty acid acyl groups. Incorporation of cholesterol (a required growth factor) into the membrane of Mycoplasma reduces the noneleotrolyte permeability and strongly suggests that at least one sterol function is stabilization of the bilayer in an optimum physical state.

The interaction of sterol and protein within membranes is poorly understood although, as stated above, sterol is believed to be excluded from the region immediately surrounding integral proteins (annulus). Dietary cholesterol has been shown to modify both structure and function of rat hepatic miorosomes, increasing the phospholipid and cholesterol content and the activities of drug metabolizing enzymes (23). Further, in work which formed the basis for this study, the ciliated organism Tetrahymena pyriformis has been shown to undergo a

compensation in the unsaturation index of the fatty acyl groups when supplied with an exogenous sterol, the slightly longer molecule ergosterol (2,24). The replacement in the cell of tetrahymanol by ergosterol results in several reproducible changes in the lipid classes. Replacement results in shortening of the average fatty acyl chain length, a decrease in the degree of unsaturation, an increase in the relative amount of 6,11-18:2 and a decrease in the amounts of both 9,12-18:2 and 6,9,12-18:3. Within the polar lipid class, the 1-alkyl 2-acyl-sn-glycero-3-(2 amino ethyl)-phosphonate and 1,2 - diacyl - sn - glycero -3- (2 amino ethyl)-phosphonate showed the greatest alteration in fatty acid composition, the glycerophospholipids, cardiolipin and phosphatidyl choline the least. A reduction in chain length and an increase in alpha hydroxy acids occured within the sphingolipids with ergosterol supplementation.

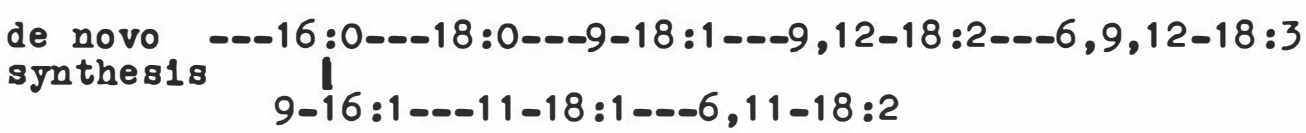
It is presumed that these alterations in the fatty acyl groups are necessary for maintenance of an optimum membrane state. Other investigators (25) have shown that similar alteraticns take place in Tetrahymena under the influence of temperature manipulation, suggesting again the presence of a mechanism to preserve an optimum

membrane state under varying environmental conditions. The particular effect investigated in this study involved the previously reported change in number and position of the double bonds of the fatty acyl groups following ergosterol supplementation (2). Erwin and Block (26) have shown that the majority of long chain fatty acids are synthesized by Tetrahymena via the following pathway:



Recently Connor et al. (27) have characterized the existence of a second, quantitatively minor pathway leading from 16:0 to the novel lipid 6,11-18:2:

Overall then, the pathway leading from de novo synthesis to the medium-to-long chain unsaturated fatty acids may be represented diagramatically as follows:



The above mentioned study by Ferguson (2) in which cells supplemented with ergosterol had smaller amounts of 9,12-18:2 and 6,9,12-18:3 and increased amounts of 6,11-18:2 lends itself to several speoulations as to the

mechanism by which the shift is accomplished, as will be discussed in a later section. However, the basis of this study is the rather unique idea of a protein-sterol interaction, one in which the presence of ergosterol, rather than tetrahymanol, in the membranes of the endoplasmic reticulum influences the activity of one or more of the enzymes involved in the elongation and desaturation process. ^{14}C-labeled octadecenoic acid, cis-9-18:1, intermediate in the major pathway, was chosen as the experimental substrate because no conversion of 9-18:1 to 6,11-18:2 is possible (27). Presuming that a sterol-enzyme influence exists and utilizing experimentally paired supplemented and nonsupplemented cultures, one might predict that a lesser amount of desaturation products (9,12-18:2 and 6,9,12-18:3) would be recovered following an incubation period with the labeled substrate from ergosterol cultures as compared to tetrahymanol (non-supplemented) cultures due to the presence of the foreign sterol in the membrane of the endoplasmic reticulum.

Several biosynthetic pathways for fatty acids have been reviewed and related to phylogenetic level by Erwin and Block (28). In addition, the fatty acid synthetase and subsequent elongation pathways have been

characterized by Jaworski et al. in plants (29) and shown to have separate requirements and characteristics (i.e. pH and heat lability). Acyl transferase activity in lymphocytes is also known to respond to stimulation by concanavalin A (30), and is inhibited by 9-tetrahydrocannabinol (31). In general then, several possibilities exist for the type and location of a homeostatic membrane control in the oell: at the microsomal site of elongation and desaturation activity; at the acyl transferase level of insertion of fatty acids into phospholipid; at the level of transport from the synthetic site to the membrane, and at the level of insertion and removal of phospholipids at the membrane. The first of these suggestions implies a direct sterol-enzyme relation, possibly through alteration of the microenvironment of an enzyme or enzymes within the endoplasmic reticulum. Sterol is found in this membrane in molar ratios to phospholipid of 0.03 to 0.08 (32). Thus, the incorporation of the slightly dissimilar true sterol, ergosterol, might alter the activity of an enzyme within the fatty acid synthesis pathway and account for the changes in fatty acid composition of sterol supplemented cells as previously characterized. A decrease in the amount of desaturation products recovered from sterol

supplemented cells following incubation with ^{14}C-labeled octadecanoate could not elucidate the exact mechanism through which sterol influences the enzyme(s) but should future microsomal incubations parallel whole cell studies the level of influence (i.e. at the microsomal site) would be known, illustrating a novel relation between these two membrane components, protein and sterol.

The goals of this research were:

1) To develop techniques required for investigating the sterol compensation effect in Tetrahymena using ^{14}C-labeled substrate (9-18:1) occupying a position intermediate in the pathway leading from 16:0 to 18:3.

2) To determine what, if any, effect sterol substitution has on the activity of desaturases toward this substrate under several conditions of growth and temperature.

3) To view the results in terms of membrane theory and previous similar work investigating the manner in which membrane composition self regulates.

Materials and Methods:

Cultures of Tetrahymena pyriformis - W were grown under sterile conditions in proteose peptone medium (2% proteose peptone, 0.1% yeast extract, Fe^{+++}-EDTA complex (90 micromolar), distilled water to a total volume of 500 ml). Culture size was 5 ml (6 ml initial if protein analysis was performed) or 18 ml (20 ml initial), in 25 ml and 50 ml Erlenmeyer cheesecloth/cotton stoppered flasks respectively. Growth temperature was maintained at either 20^{o} or 29.5^{o}. Cultures were inoculated from stock 'starter' 29.5^{o} cultures maintained in 2.5 ml medium. Volumes of starter cells added and growth times were regulated to insure that incubation with substrate would take place during log growth phase (ie. for 18 ml cultures grown at 29.5, addition of .4 ml starter 12 hrs. prior to incubation, or 0.1 ml starter 16-18 hrs. prior to incubation). Cultures were maintained before and during incubations in a constant temperature incubator (Forma Scientific). To each culture 10-15 microliters of ^{14}C-labeled oleic acid (New England Nuclear Co.) were added as a solution in absolute ethanol ($0.5-1.0 \times 10^{6}$ counts per minute per 10 microliters). Incubation times are described in Results. Ergosterol also was added as an ethanol

solution at the time of inoculation unless otherwise indicated. Equivalent amounts of ethanol were added as a control to non supplemented cultures. Two methods of lipid extraction following incubation were used. Initially reactions were stopped and extraction carried out by the method of Folch (33). The lower organic layer was taken to dryness by rotary evaporation and the lipid extract transfered to screw capped vials as a solution in 2-4 ml chloroform methanol 2:1 (v:v). An alternate method was used in later experiments. Cells were harvested by filtration of the culture through 8.0 micrometer Millipore filters (Millipore Corp.). Lipids were then extracted by placing the filter in 20 ml CM 2:1 for 5 minutes. After removing the filter the solvent was removed by rotary evaporation and the lipid extract transfered to screw capped vials as above.

Fatty acid methyl esters were prepared from acyl lipids by addition of 1-2 ml of a 5% HCl/CH_3OH solution to the lipid extract following evaporation of the solvent under nitrogen. Vials then were flushed with nitrogen and placed in a heating block at 75^{o} for 60 minutes. After cooling the samples, one ml of water was added and the methyl esters were extracted twice into a total of four ml petroleum ether. Solvent was removed by

evaporation under a nitrogen stream.

Argentation chromatography was used to separate the fatty acid methyl esters according to chain length and number of double bonds. Methyl esters were applied as a solution in 15% benzene/petroleum ether to a 0.5 gm silver nitrate-impregnated Unisil (20% $AgNO_3$ w/w) column and the methyl esters were eluted with increasing proportions of benzene in petroleum ether followed by one elution with acetone, required to remove all of the 6,9,12-18:3 from the column. A control column (substrate + 5 mg whole lipid) was used to correct the percent of counts per minute recovered as 18:2 or 18:3 in experimental samples. Two systems are described and the fatty acid methyl esters contained in each eluate listed, as determined by gas liquid chromotography using a Varian Aerograph #286010-00 (Varian Corp.) with a four foot glass column containing 15% HI-EFF-BP (DGS) on Chromosorb P (80-100 mesh) and operating column temperature of 160°. Many other systems were investigated during the course of the work (data not shown) to establish a procedure yielding the required separation. The first method shown allows for separation of both 9,12-18:2 and 18:3 from the 9-18:1 substrate:

	Amount of solvent	% Benzene in petroleum ether	Peaks Identified
1)	10 ml	15%	X:0 (no double bonds ie. 16:0, 14:0)
2)	10 ml	40%	X:0, X:1 (9-18:1)
3)	10 ml	50%	X:2 (9,12-18:2)
4)	20 ml	75%	X:2 (6,11-18:2)
5)	10 ml	acetone	X:3 (6,9,12-18:3)

The second technique allows for more rapid separation of 18:3 alone:

	Amount of solvent	% Benzene in petroleum ether	Peaks Identified
1)	20 ml	40%	X:0, X:1, 9,12-18:2
2)	10 ml	75%	6,11-18:2
3)	20 ml	acetone	6,9,12-18:3

Identification by gas liquid chromatography of the methyl esters contained in the eluates was made by comparison of the retention times of the unknown peaks to that of methyl etearate or to the methyl stearate peak of Qual Mix M (C15-C20, Supelco).

One ml of each eluate was placed in a scintillation vial with five ml toluene-base scintillation fluid (6.0 gm POP, 0.1 gm POPOP-Eastman, 1 l scintillation grade toluene - Fisher) and counted for two minutes in a

Beckman LS-100C liquid scintillation counter. A background count was taken prior to counting of experimental samples by placing five ml of scintillation cocktail in an empty scintillation vial and counting for two minutes. Experimental samples were counted when it was certain that background and/or contamination did not exceed 100 counts per minute. Under these conditions no background correction was applied to experimental counts per minute. Counts per minute in each experimental aliquot were corrected for eluate volume (cpm x volume of eluate) and then expressed as percent of the total radioactivity recovered from the column. Protein analysis was performed by centrifuging a frozen and thawed sample of the culture at 40,000 x g for 20 minutes, followed by one wash of the pellet with distilled water and recentrifugation at 40,000 x g for 20 minutes in a Beckman L3-50 ultracentrifuge maintained at 4°. The supernatant was drawn off, one ml distilled water added, and the pellet was resuspended by sonication with a Sonic 300 dismembrator (Arteo Systems Inc.) equipped with a microtip, at a setting of 30% relative output. 0.3 ml of the suspension was used for protein determination by a modified biuret procedure (34).

As will be shown in Results, early experiments

(such as those using 5 ml cultures and extraction by Folch partition) had large variations in the percent of total radioactivity recovered from column chromatography preventing any meaningful comparison of the data. Therefore, modifications of the experimental procedure were examined at each step from harvesting through chromatography. For instance, at the harvesting/extraction step the Millipore filtration method was substituted for the Folch partition sinoe recovery of total cpm from the column was less variable with filtration. Preparation of fatty acid methyl esters was accomplished using several procedures in addition to the 5% HCl/CH_3OH. Alkaline hydrolysis followed by or prior to treatment of the samples by diazomethane was investigated and discarded as a method since it yielded no less and possibly greater amounts of apparently unconverted free fatty acid contaminants.

The procedure eventually found to yield the most consistent data utilized 18 ml cultures, millipore filtration, methylation with HCl/CH_3OH and column chromatography as previously described.

Duration of storage of lipid samples was minimized as much as possible within experiments and all samples, when stored, were kept as CM 2:1 solutions under

nitrogen at 0^{o} to prevent oxidation of the double bonds.

Ergosterol (mp. 162-164^{o}, Sigma Chemical Co.) was recrystalized from methanol. Unisil (100-200 mesh, Clarkson Chemical Co.) was washed with methanol and stored in a 100^{o} oven until silver nitrate impregnation. The prepared $AgNO_3$-Unisil was stored in the dark at 100^{o} to avoid hydration and photoreactions.

Results

Although some preliminary work was done with microsome preparations and ^{14}C-labeled palmitate, it became apparent that whole cell studies were necessary before microsomal preparations would be useful and that oleate was a better overall substrate for desaturation. Oleate may be used to examine both the 6- and 12-desaturases at the end of the major pathway:

16:0 - 18:0 - 9-18:1 - 9,12-18:2 - 6,9,12-18:3

A column technique was developed to isolate both products (9,12-18:2 and 6,9,12-18:3) individually from the substrate. Total recovery of counts per minute varied from about 40-70% depending on culture density and recovery technique (See Methods).

Figure 2 shows the results of incubating the ^{14}C-labeled 9-18:1 substrate for various time periods with cell cultures grown at 20°. More of the radioactive label is recovered as 18:2 than as 18:3 during the early time periods. The amount of 18:3 rises steadily throughout the experiment, while the amount of 18:2 rises and then declines reflecting the sequence of reactions taking place, that is, 9-18:1 is first desaturated to 9,12-18:2, which then serves as substrate for the 6-desaturase in the conversion of 18:2 to 6,9,12-18:3. 18:3 eventually becomes the major product as incubation times increase (Figure 2, see twelve hour time point).

The effect of substrate concentration on enzyme activity also was investigated by incubating with a combination of nonlabeled and labeled substrate, varying only the concentration of the nonlabeled 18:1. The results of these experiments (data not shown) indicated that desaturase activity is not enhanced by higher substrate concentration, since an increase in the amounts of labeled 18:2 or 18:3 did not occur at concentrations of added nonlabeled 18:1 ranging from .5 to 20 micromoles. For this reason subsequent experiments were carried out using only labeled 18:1 as substrate.

Initial experiments to investigate the effect of ergosterol supplementation on enzyme activity were performed using five ml cultures grown at 20°, approximately nine degrees below optimum growth temperature. Under these conditions the growth rate is slower than at 29° and a greater amount of highly unsaturated product is required in the membrane lipid to maintain optimum fluidity (25,35). Table I lists the results of these experiments for ergosterol concentrations of 5, 10, 15, 20, and 40 mg/500 ml culture medium, in which cells were grown either with or without the sterol supplement prior to incubation with substrate. Percentages indicate the percent of the total counts per minute recovered from

column chromatography following extraction of lipids by the Folch partition method and methylation by HCl/CH_3OH as described in Methods. Although higher ergosterol concentrations appear to influence the amounts of labeled 18:2 and 18:3 recovered, the data are inconsistent and statistically insignificant when applied to a paired T test. Further, since the differences in static lipid composition characterized by Ferguson (2) oooured at ergosterol oonoentrations of 5 mg/500 ml, it seemed desirable to investigate the hypothetical enzyme regulation at that concentration. A similar procedure was performed at 29.5° (optimum growth temperature) and the results are listed in Table 2. Again, the results were widely variable, as indicated by the percent difference of labeled 18:3 recovered for supplemented vs nonsupplemented cultures, and no significant effect of ergosterol concentration could be demonstrated.

To investigate the possibility that the experimental variability might correlate to culture density, in several of the experiments described above, the initial culture volume was increased to six ml so that a protein assay might be performed. One ml of culture was removed prior to addition of substrate and a total protein determination was made on cells from each culture

(see Methods). Following separation of the labeled 18:2 and 18:3, the percent recovery value for 18:2 and 18:3 was divided by the protein value to express recovery of the label as either %18:2/mg cell protein or %18:3/mg cell protein. In no case did this procedure alter the variability of the data since cell protein values were routinely very similar for culture pairs (supplemented vs nonsupplemented).

At this point a procedural modification was made in an attempt to provide more consistent data. Incubation culture size was increased from five ml to twenty ml (18 ml at incubation if protein determination was performed), in an attempt to facilitate more uniform and consistent dispersion of the insoluble ergosterol into the aqueous medium. Experiments were then performed by growing cell cultures in nonsupplemented medium at 29.5° until log growth stage was reached. At that time ergosterol solution (supplemented) or ethanol (control) was added to the culture. Sixty minute incubations were performed with cultures exposed to the supplement for zero, one, two, three, four, five, and six hours. Control samples were examined at these times also. This procedure was adopted to allow examination of the effect of ergosterol on desaturase activity

during the time of its initial incorporation into the cell membranes. Ergosterol appears in the microsomal fraction within 1 - 2 hours after its addition to the cultures (Ferguson, personal communication). The results of these experiments are shown in Table 3. Again the data are variable and no positive or negative trend can be established. Correcting for culture protein values, as described above, again did not alter the results.

One experiment was performed to examine the effect of long-term incubation with substrate. The ^{14}C-labeled substrate and ergosterol solution (supplemented) or ethanol (nonsupplemented) were placed in the medium at the time of inoculation of the cultures. Growth occured until log growth stage was reached (12 hours). The results of this procedure showed that large amounts of radioactive label were recovered as 18:3 and there was no difference between culture types, as might be expected when a limited amount of substrate was used for extensive membrane formation during long term culture growth. It also demonstrates, again, that 18:2 serves as substrate for conversion to 18:3. (Table 4)

Modification of the experimental procedure was again made. In substitution for the Folch partition, cells were harvested following incubation with substrate

by millipore filtration and the lipids were extracted from the filter into twenty ml of chloroform-methanol 2:1 (v:v). This method routinely gave more consistent total recovery of radioactive material. Either cells were grown for 12 hours with or without ergosterol at 29.5° and subjected to a temperature change to 20° at the time of incubation with substrate (Table 5), or they were grown in unsupplemented medium and subjected to a temperature change to 20° during incubation following a two hour exposure to ergosterol or ethanol (Table 6). In these experiments the average values for supplemented and control cultures were similar and variation within individual pairs was somewhat reduced. However, the difference between culture pairs (supplemented vs control) was too small to indicate any effect of ergosterol on desaturase activity. In summary then, to this point no experimental procedure had demonstrated a reproducible significant effect of ergosterol on the desaturation of the ^{14}C-labeled oleate.

Since several individual experiments had indicated a slightly lesser degree of desaturation of 18:1 for supplemented cell cultures, and the most consistent trend had been seen at 20° (Table 1), albeit at higher ergosterol concentrations, all of the following experi-

ments were performed at 20°. At this temperature less membrane production is ooouring per unit time and, as stated earlier, a greater amount of highly unsaturated lipid is required. For these reasons, small differences in enzyme aotivity might be magnified and detected at 20° using the procedural modifications previously listed (larger culture volume and millipore filtration). Table 7 lists data from five experiments performed with 20° cultures grown with or without ergosterol supplement from the time of inoculation. They are listed in order of increasing estimated cell density at the time of incubation with ^{14}C -substrate. Care was taken to carry out all procedures by strict pairing of a supplemented with a nonsupplemented culture to minimize processing effeots. For example, cultures listed as Tol 1 (nonsupplemented) and Eol 1 (supplemented) were supplied with substrate and, following incubation, extracted at the same time, rather than carrying out these procedures on all of the nonsupplemented followed by all the supplemented cultures. The data listed in the Table show that in all cases the nonsupplemented cultures had a greater conversion of ^{14}C-labeled 18:1 to both 18:2 and 18:3 than did the ergosterol supplemented culture, and the lowest nonsupplemented value observed was larger

than the highest value for the supplemented cultures. Table 8 shows a summary of these data with a statistical analysis utilizing a paired T test. This analysis, based on a rejection of the hypothesis that there be no difference between desaturase activity of the two culture types, indicates a T value greater than that required for 99.5% rejection for both 18:2 and 18:3. The rejection of the hypothesis means therefore that there is a statistically significant difference between the recoveries of labeled 18:2 and 18:3 following incubation with ^{14}C-9-18:1 from supplemented and nonsupplemented cultures grown at 20^{o} with long term exposure to ergosterol.

Discussion

Many lines of evidence have been advanced in support of the theory that change in the fatty acid composition of cellular membranes oocurs as a regulatory phenomenon through which membrane homeostasis is maintained in eucaryotic (36,37) as well as procaryotic systems (2, 22, 35, 38, 39, 40). However, the exact mechanism involved in fatty acid regulation remains elusive and oould conceivably involve phospholipid flip-flop within the membrane, insertion and removal of lipid from the membrane, transportation from or at the site of lipid synthesis (endoplasmic reticulum). In Tetrahymena certain controversy does exist, notably over whether alteration of polar head composition takes place (2,41) and over the failure by some investigators to report the existence of the 6,11-isomer of linolenate (39). Nontheless, the concept of such a regulatory mechanism involving fatty acid composition seems reasonable in that it provides for optimum membrane function in a homeostatic state of interaction with the environment.

Although fatty acid regulation has been examined with a variety of experimental variables including temperature (38, 39) and diet (36, 41), the fact that

membrane bound sterol is also involved, possibly in a feedback regulatory role with fatty acid synthetic enzymes, may have far reaching implications when considered with respect to higher organisms. Mitropoulos et al. (42) have recently directed attention to such a sterol-enzyme concept by demonstrating an activity dependence of 3-Hydroxy-3-Methylglutaryl Coenzyme A reductase (HMG-CoA reductase) on the concentration of free cholesterol in rat liver microsomal preparations, proposing that the size of the cholesterol pool surrounding this enzyme is important to intracellular HMG-CoA reductase regulation. Thus sterol may, in fact, contribute to a variety of critical biochemical events not yet characterized, by interaction with other components, notably enzymes, contained within membranes.

Recently Sprecher (43) has suggested several possible points of regulation in the long chain fatty acid biosynthetic pathways of rat liver cells, involving both desaturase and elongation enzymes. Tetrahymena differs from such eucaryotic systems, in part, in that no essential fatty acids are required for growth due to the presence of an active 12-desaturase in the major pathway providing substrate for further desaturation to the required structure. In addition to 9,12-18:2 at least

two other substrates exist for the 6-desaturase: 9-18:1 (leading to the minor product 6,9-18:2) and 11-18:1 (leading to 6,11-18:2). Since 6,11-18:2 is found only in the minor synthetic pathway it offers both a second possibility for examining the effect of ergosterol on the activity of the 6-desaturase enzyme and the advantage that only one reaction takes place, that is, insertion of the double bond at the Δ^6 position. The effect of ergosterol supplementation on this reaction is currently being investigated (Ferguson, personal communication). In the present study both the 6- and 12-desaturases are involved and one or both may be influenced by the presence of ergosterol in the cell membranes. An extension of the study presented is suggested using ^{14}C-labeled 9,12-18:2, which would isolate the 6-desaturase for examination.

The data presented in Table 7 indicate the desaturation by ergosterol supplemented cultures to range from 54 - 86% of that of nonsupplemented cultures with respect to 18:2 and from 36 - 64% with respect to 18:3, as determined by the amount of radioactive label recovered as the two compounds. The percent increases with increasing estimated cell density. One explanation for this is that the sterol also serves as a food source

for the cells and supplemented cultures may grow at a slightly faster rate.

One additional procedure was performed to be certain that the difference in recovery of radioactive label between supplemented and nonsupplemented cultures was not due to large cell density differences. In two experiments one ml of culture was removed from each flask prior to incubation with substrate. This one ml aliquot was diluted to approximately 10 ml with phosphate buffer (pH - 7.4) and 1 - 2 drops of crystal violet added. After a few minutes staining time, the solution was filtered by Millipore filtration and a section of the filter membrane placed on a glass slide in several drops on immersion oil. Ten to fifteen fields were counted under low power light microscopy and the average number of cells for each culture type compared. This technique showed, as had culture protein determinations done in earlier experiments, that the densities of the cultures were nearly identical.

Many experiments were performed during the course of this study in the process of refining techniques and investigating conditions. Those thought to represent advances and/or as demonstrating particular problems encountered have been presented.

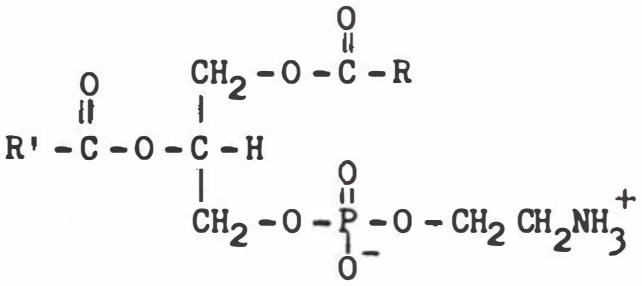
Although a good deal of further investigation will be required before it can be stated with certainty that a sterol-desaturase relation exists as a regulatory mechanism in Tetrahymena, the results of this work are encouraging. Experiments using other substrates in the biosynthetic pathways and in vitro assays are now indicated. Ultimately it may be possible to demonstrate that this hypothetical enzyme regulation by sterol is an actual event in membrane physiology.

FIGURE 1

SOME LIPIDS FOUND IN *TETRAHYMENA PYRIFORMIS*

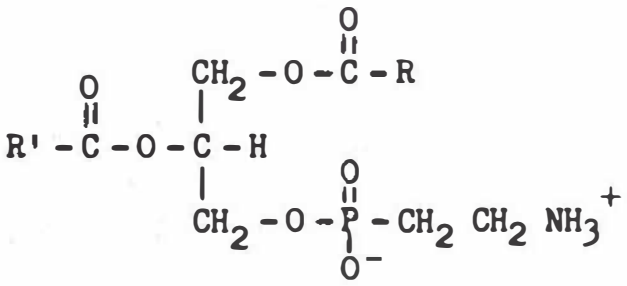
FIGURE 1

SOME LIPIDS FOUND IN TETRAHYMENA PYRIFORMIS



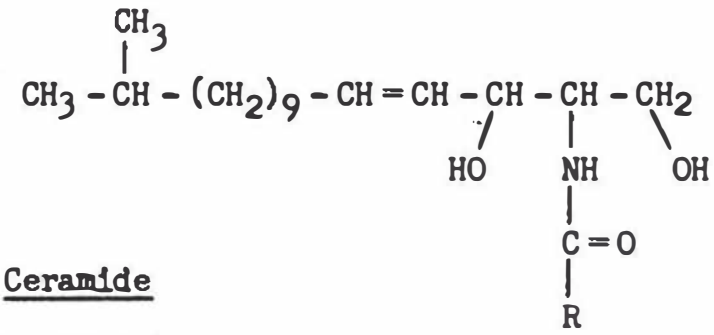
Ethanolamine Phospholipid

(Glycerophospholipid)



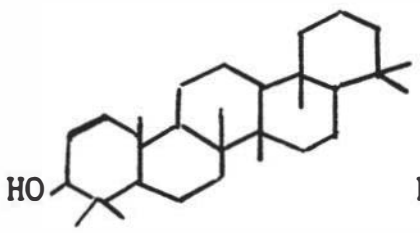
Ethanolamine Phosphonolipid

(Phosphonolipid)

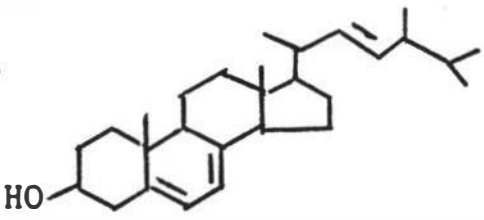


Ceramide

(Sphingolipid)



Tetrahymanol



Ergosterol

FIGURE 2

THE EFFECT OF TIME OF SUBSTRATE INCUBATION (^{14}C-9-18:1) WITH *TETRAHYMENA* CULTURES ON THE PERCENT RECOVERY OF ^{14}C-LABELED 9,12-18:2 AND 6,9,12-18:3[a].

a. Cultures were grown in nonsupplemented proteose-peptone medium to log growth stage prior to addition of substrate. Values are expressed as the percent of total counts per minute recovered from $AgNO_3$-Unisil column chromatography (see Methods). Each time point represents 2-3 determinations. ■ - ■ 9,12-18:2, ● - ● 6,9,12-18:3, ▲ - ▲ 18:2 + 18:3.

Figure 2

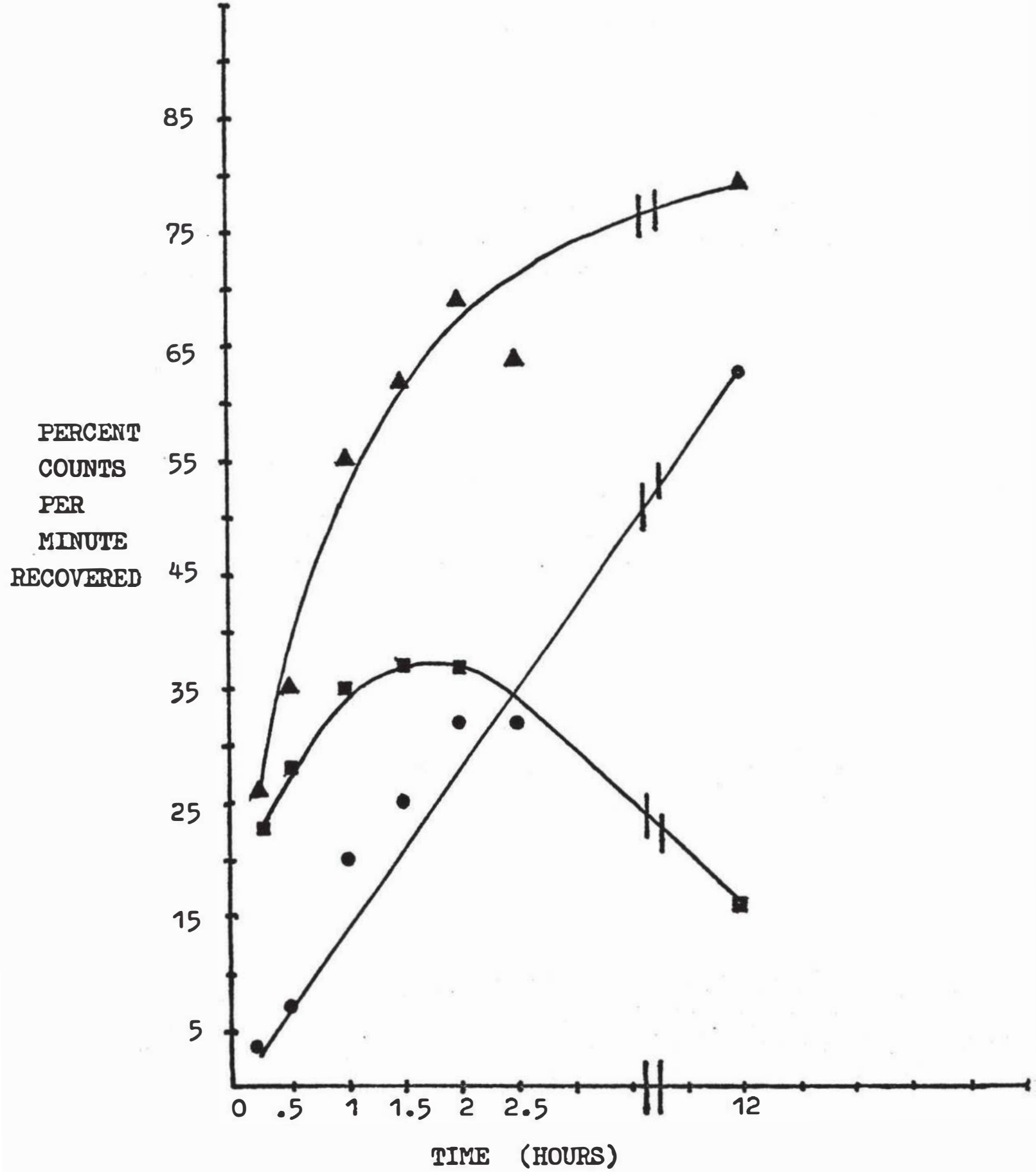

85
75
65
55
45
35
25
15
5
PERCENT
COUNTS
PER
MINUTE
RECOVERED
0
.5
1
1.5
2
2.5
12
TIME (HOURS)

TABLE 1

PERCENT OF TOTAL COUNTS PER MINUTE RECOVERED AS 9,12-18:2 AND 6,9,12-18:3[a] FOR NORMAL (TOL) AND OELLS SUPPLEMENTED WITH ERGOSTEROL (EOL). GROWTH TEMPERATURE 20°.[b]

ERGOSTEROL CONCENTRATION	% 9,12-18:2[c]			% 6,9,12-18:3		
	TOL	EOL	(EOL-TOL)	TOL	EOL	(EOL-TOL)
5 mg/500 ml	15	17	+2	16	21	+5
	16	13	-3	15	15	0
10 mg/500 ml	22	18	-4	17	15	-2
15 mg/500 ml	22	11	-11	11	9	-2
20 mg/500 ml	17	10	-7	11	9	-2
25 mg/500 ml	17	9	-8	15	8	-7

a. Values are expressed as the percent of total counts per minute recovered following a one hour incubation with ^{14}C-labeled 9-18:1, extraction of lipids by the method of Folch (33) and methylation with HCl/CH_3OH (see Methods).

b. Cells were grown in 5 ml medium supplemented with ergosterol or an equivalent amount of ethanol at the time of inoculation of the culture with starter cells.

c. Eaoh value represents one determination. The percent difference of recovery from supplemented and nonsupplemented cultures is listed at the right (EOL-TOL).

TABLE 2

PERCENT OF TOTAL COUNTS PER MINUTE RECOVERED AS 6,9,12-18:3[a] FROM NORMAL CELLS (TOL) AND CELLS SUPPLEMENTED WITH ERGOSTEROL (EOL).[b] GROWTH TEMPERATURE 29.5°.

ERGOSTEROL CONCENTRATION	% 6,9,12-18:3[c] TOL	EOL	(EOL-TOL)
5 mg/500 ml	26	25	-1 (3)
	23	31	+8 (2)
10 mg/500 ml	32	32	0 (3)
	28	22	-6 (2)
20 mg/500 ml	20	27	+7 (3)

a. Values are expressed as the percent of total counts per minute recovered from column chromatography following incubation of five ml cultures with ^{14}C-labeled 9-18:1. Incubation time (hours) is shown in parenthesis. Extraction of lipid was performed by the method of Folch (33) and methylation with HCl/CH_3OH (see Methods).

b. Cells were grown in 5 ml medium supplemented with ergosterol or an equivalent volume of ethanol at the time of inoculation of the culture with starter cells.

c. Eaoh value represents one determination. The percent difference of recovery from supplemented and nonsupplemented cultures is listed to the right (EOL-TOL).

TABLE 3

PERCENT OF TOTAL COUNTS PER MINUTE RECOVERED AS 9,12-18:2 AND 6,9,12-18:3[a] FOR NORMAL (TOL) AND SUPPLEMENTED (EOL) CELL CULTURES FOLLOWING INCUBATION WITH ^{14}C- 9-18:1 AT VARIOUS TIMES AFTER ADDITION OF ERGOSTEROL OR ETHANOL TO THE GROWTH MEDIUM.[b] GROWTH TEMPERATURE 29.5°.

TIMES ^{14}C-SUBSTRATE ADDED AFTER ERGOSTEROL (HOURS)	% 9,12-18:2[c]			% 6,9,12-18:3		
	TOL	EOL	(EOL-TOL)	TOL	EOL	(EOL-TOL)
0	21	24	+3	21	16	-5
	30	28	-2	22	21	-1
	26	25	-1	22	19	-3
	(26)	(26)	(0)	(22)	(19)	(-3)
1	12	12	0	20	16	-4
	26	26	0	13	15	+2
	26	23	-3	17	15	-2
	(21)	(20)	(-1)	(17)	(15)	(-1)
2	22	15	-7	18	18	0
	26	26	0	13	13	0
	26	29	+3	20	22	+2
	(25)	(23)	(-1)	(17)	(18)	(+1)
3	19	24	+5	11	22	+11
	22	27	+5	9	11	+2
	24	27	+3	17	20	+3
	(22)	(26)	(+4)	(12)	(18)	(+5)

TABLE 3 (CONTINUED)

4	20	12	-8	16	11	-5
5	12	12	0	8	11	+3
	14	14	0	25	22	-3
	(13)	(13)	(0)	(17)	(17)	(0)
6	15	12	-3	26	24	-2
	11	11	0	15	14	-1
	(13)	(12)	(-2)	(21)	(19)	(-2)

a. Values are expressed as the percent of total counts per minute recovered from column chromatography following a sixty minute incubation of 18 ml cultures with substrate (9-18:1), extraction of lipid by the method of Folch (33) and methylation with HCl/CH_3OH (see Methods).

b. Cells were grown in nonsupplemented medium to log growth stage at which time ergosterol (5 mg/500 ml) or an equivalent volume of ethanol was added to the medium. Substrate was added following sterol addition at the times indicated.

c. Each value represents one determination. The average value is underlined in parenthesis at the bottom of each time period. The percent difference of recovery from supplemented and nonsupplemented cultures is listed to the right (EOL-TOL).

TABLE 4

THE EFFECT OF LONG TERM INCUBATION AT 29.5° WITH ^{14}C-9-18:1 ON THE RECOVERY OF LABELED 18:2 AND 18:3 FROM CULTURES GROWN IN THE PRESENCE (EOL) OR ABSENCE (TOL) OF ERGOSTEROL.[a]

PERCENT[b]	TOL	EOL
9,12-18:2	18	13
	16	13
6,9,12-18:3	63	63
	63	62

a. Cells were grown for twelve hours at 29.5° in 20 ml medium containing ^{14}C-labeled 9-18:1 substrate or substrate plus ergosterol (5 mg/500 ml). Extraction was carried out by the Millipore filtration method and methylation with HCl/CH_3OH (see Methods).

b. Values are expressed as the percent of total counts per minute recovered from column chromatography. Each value represents one determination.

TABLE 5

THE EFFECT OF LONG TERM EXPOSURE TO ERGOSTEROL ON THE PERCENT RECOVERY OF LABELED 9,12-18:2 and 6,9,12-18:3 WHEN TEMPERATURE IS DECREASED FROM 29.5° to 20° AT THE TIME OF SUBSTRATE INCUBATION.[a]

EXPERIMENT NUMBER	PERCENT RECOVERY[b] TOL 1[c]	EOL 1	TOL 2	EOL 2	TOL 3	EOL 3	AVERAGE TOL	AVERAGE EOL
1								
(18:2)	28	33	34	31	31	28	31	31
(18:3)	17	18	18	20	17	19	18	19
2								
(18:2)	35	30	35	33	37	29	36	31
(18:3)	26	18	21	21	24	16	24	18
3								
(18:2)	22	22	25	20			24	21
(18:3)	22	22	20	25			21	24

a. Cells were grown at 29.5° in 20 ml unsupplemented medium (TOL) or medium containing ergosterol (5 mg/500 ml)(Eol) to log growth stage. Incubation occured for sixty minutes, followed by harvesting and extraction by the Millipore filtration method and methylation by HCl/CH_3OH (see Methods).

b. Values are expressed as the percent of total counts per minute recovered following column chromatography. Each value represents one determination. Average values for each experiment are listed to the right.

c. Values are listed within each experiment by paired cultures (ie TOL 1, EOL 1)(see Results).

TABLE 6

THE EFFECT OF A 2 HOUR EXPOSURE TO ERGOSTEROL ON THE PERCENT RECOVERY OF LABELED 9,12-18:2 AND 6,9,12-18:3 WHEN TEMPERATURE IS DECREASED FROM 29.5° TO 20° AT THE TIME OF SUBSTRATE INCUBATION.[a]

	PERCENT RECOVERY[b]						AVERAGE	
RECOVERY OF:	TOL 1[c]	EOL 1	TOL 2	EOL 2	TOL 3	EOL 3	TOL	EOL
(18:2)	24	16	19	19	17	15	20	17
(18:3)	8	15	13	12	8	15	10	14

a. Cells were grown at 29.5° in 20 ml unsupplemented medium to log growth stage, at which time ergosterol (5 mg/500 ml) (EOL) or ethanol (TOL) was added to the medium. A sixty minute incubation with substrate occured two hours following addition of sterol or the control. Extraction of lipid was accomplished by the Millipore filtration method and methylation with HCl/CH_3OH (see Methods).

b. Values are expressed as the percent of total counts per minute recovered following column chromatography. Each value represents one determination. Average volues for each experiment are listed to the right.

c. Values are listed by paired cultures (see Results).

TABLE 7

THE EFFECT OF ERGOSTEROL ON THE PERCENT RECOVERY OF LABELED 9,12-18:2 and 6,9,12-18:3 AS COMPARED TO NONSUPPLEMENTED CULTURES GROWN AT 20°.[a]

EXPERIMENT NUMBER		TOL 1[b]	TOL 2	TOL 3	EOL 1	EOL 2	EOL 3	$\frac{\bar{X}\ EOL}{\bar{X}\ TOL}$[c]
1	18:2	15.9	12.7	25.7	10.7	10.7	7.6	$\frac{9.7}{18.1} = 54\%$
	18:3	4.4	4.2	4.5	2.5	1.4	1.0	$\frac{1.6}{4.4} = 36\%$
2	18:2	26.6	33.3		19.8	15.4		$\frac{17.6}{30.0} = 59\%$
	18:3	7.7	10.2		3.5	4.6		$\frac{4.1}{9.0} = 46\%$
3	18:2	30.7	30.0	27.2	20.1	20.0	22.8	$\frac{21.0}{29.3} = 72\%$
	18:3	15.5	14.8	14.5	8.4	8.3	8.2	$\frac{8.3}{14.9} = 56\%$
4	18:2	41.1	39.2	40.0	24.0	20.5	22.5	$\frac{22.3}{40.1} = 56\%$
	18:3	15.8	16.7	14.7	11.9	6.5	8.4	$\frac{8.9}{15.7} = 57\%$
5	18:2	35.0	35.4	36.8	30.4	33.0	29.1	$\frac{30.8}{35.7} = 86\%$
	18:3	25.7	20.9	24.0	17.8	11.2	15.9	$\frac{15}{23.5} = 64\%$

a. Cells were grown at 20° in 20 ml unsupplemented medium (TOL) or medium supplemented with ergosterol (5 mg/500 ml)(EOL) Incubation with ^{14}C-9-18:1 occured for 60 minutes, followed by harvesting and extraction by the Millipore filtration method and methylation by HCl/CH_3OH (see Methods).

TABLE 7 (CONTINUED)

b. Values are expressed as the percent of total counts per minute recovered following column chromatography. Each value represents one determination.

c. Data is summarized in Table 8.

TABLE 8

SUMMARY OF DATA PRESENTED IN TABLE 7.[a]

EXPERIMENT NUMBER		TOL-EOL		TOL-EOL
1	18:2	5.2	18:3	1.9
		2.0		2.8
		18.1		3.5
2		6.8		4.2
		17.9		5.6
3		17.1		3.9
		18.7		10.2
		17.5		6.3
4		4.6		7.9
		2.4		9.7
		7.7		8.1
5		10.6		7.1
		10.0		6.5
		4.4		6.3
		$\bar{X}\ \frac{143}{14} = 10.2$		$\bar{X}\ \frac{84.0}{14} = 6$
		$S = \pm 6.39$		$S = \pm 2.5$
		$t = 5.9725$		$t = 8.98$

a. See footnotes Table 7.

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