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Tiee-Chyau Miin
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ERGOSTEROL EFFECT ON THE DESATURATION OF ^{14}C-CIS-VACCENATE IN TETRAHYMENA

(TITLE)

BY

Tiee-Chyau Miin

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

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IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
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ERGOSTEROL EFFECT ON THE DESATURATION OF ^{14}C-CIS-VACCENATE IN TETRAHYMENA

Thesis Approved

Dr. K. A. Ferguson

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Dr. D. H. Buchanan

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371814

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Terms and Abbreviations

1. fatty acids

   a,b-X:c -- a,b (position of double bonds); X (carbon chain length); c (number of double bonds). Example: 6,11-18:2 is a fatty acid with 18 carbons and 2 double bonds at carbons 6 and 11.

2. C:M 2:1 -- chloroform:methanol 2:1 (v/v)

Abstract

Supplement of ergosterol to the growth medium of the ciliated protozoan Tetrahymena pyriformis W leads to incorporation of the foreign sterol within cell membranes and suppression of synthesis of the native sterol-like 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 added into the phospholipid bilayer of the membranes.

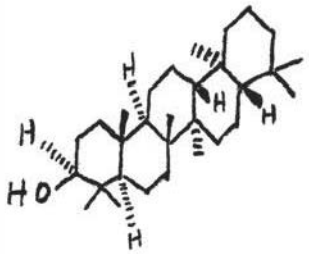
The present study, using several different conditions of growth temperature, substrate concentrations and incubation time, and ergosterol concentrations and exposure time, is an attempt to provide evidence supporting a hypothetical regulatory mechanism. This mechanism proposes that there is a feedback regulation by membrane-bound sterol on an enzyme or enzymes involved in synthesis of the long chain fatty acids contained in membrane phospholipid. Such a mechanism could account for the balance between sterol and fatty acid content of membrane. The data presented here show that a statistically significant increase in desaturation of ^{14}C-cis-vaccenate can be demonstrated in Tetrahymena cell cultures whose membranes contain the foreign sterol, when growth temperature is maintained at 20^{o} or 29.5^{o}.

Tetrahymena desaturated ^{14}C-cis-vaccenate substrate in both ergosterol supplemented and normal cultures. The ^{14}C labeled product, 6,11-18:2 was recovered and separated by silver nitrate-Unisil column chromatography.

Introduction

As the function of cholesterol in human membranes is being studied around the world (1), another sterol (ergosterol) is studied in our laboratory to elucidate whether it can influence fatty acid biosynthesis in Tetrahymena pyriformis W. The goal of this research is to find out part of the function of the sterol in the biosynthesis of lipids in Tetrahymena, and it is hoped that this finding can provide information about the function of cholesterol in humans.

Tetrahymena pyriformis W is a pearshaped ciliated protozoan. It synthesizes a natural sterol-like pentacyclic triterpene solid alcohol, tetrahymanol. Tetrahymanol has the structure:



Tetrahymanol, which comprises 0.14% of the dry weight of these cells (2), is found in membranes such as cilia (3, 4), microsomes (3), etc; and in the molar ratio to phospholipids of 0.5 and 0.041 respectively in these membranes.

Just as cholesterol, a tetracyclic triterpene, is found in human cell membranes, tetrahymanol is the only sterol-like molecule found in Tetrahymena membranes. Tetrahymena itself can not synthesize any true sterols (cholesterol, ergosterol etc), but it can incorporate them (5, 6). Tetrahymanol and phospholipids are the most plentiful lipids found in membranes; they associate with membrane proteins by both hydrophobic and hydrophilic interactions. The most widely

accepted model of the biological membrane is the fluid mosaic model of S. J. Singer (7, 8).

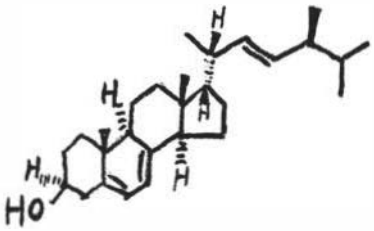
This model says that phospholipids, which are the major membranous lipids, form a bilayer: their hydrophilic heads (phosphogroup) constitute the top and bottom surfaces of the membrane and can associate with peripheral proteins by hydrophilic interactions, while the hydrophobic tails (extended chains of fatty acyl groups) are buried in the membrane interior and can interact with integral proteins by hydrophobic (or van der Waals) interaction. The total thickness of the membrane is about 45 Å. Tetrahymanol is mostly hydrophobic and has only one hydrophilic group (hydroxyl) and therefore is mostly buried in the membrane interior. The interior of the membrane has larger freedom of movement compared with the exterior, due to the weaker van der Waals interactions in the interior compared to the exterior ionic or hydrogen bonding interactions. Proteins, which contribute to the structural integrity of the membrane, can act as enzymes or function as pumps (moving material into and out of cells and organelles). It is the diversity of its protein activity that gives each particular membrane its distinctive character.

One of the important properties of any membrane is its fluidity. In chemical terms, a membrane can be considered to be a nonhomogeneous mixture of lipids (phospholipids, sterols or sterol-like substances, carotenoids, sphingolipids, and phosphonolipids) and proteins (including enzymes). It doesn't have a very sharp melting point. In biochemical terms, the physical state of the membrane above the melting point is liquid-crystalline and the phase below melting point is gel. Two factors determine this fluidity (gel or liquid-

crystalline): the chemical composition of membrane, and the surrounding temperature ([9]). The former includes the extent of saturation of the lipid tails (fatty acids), the amount and chemical structure of the sterols, and the kind of polar group. Any changes in these factors might cause change of fluidity, and the cells should do some self-regulation to compensate for these changes if they intend to maintain their natural fluidity. A direct input of the sterol (ergosterol, for example) at the fatty acid biosynthetic level might represent an efficient means of regulation of the amounts of suitable fatty acid products (cis-vaccenic acid, for example) to preserve the natural fluidity of membranes.

Ergosterol is used in this research to attempt to detect a regulation of the biosynthesis of fatty acids in microsomal membranes.

Ergosterol is the well-known sterol of fungi and yeasts, which functions as a precursor of Vitamin D ([10]), and has the structure:



It is obvious that ergosterol and tetrahymanol have some differences in molecular size and shape (one ring, one side chain, three double bonds and some methyl groups).

It is logical to postulate that this different shape and size of the ergosterol molecule would lead to different interactions with phospholipids in Tetrahymena membranes. This would cause cells to have either

different membrane properties (different fluidity, for example) or altered, compensating fatty acid compositions when tetrahymanol was replaced by ergosterol.

It has been found that ergosterol can be incorporated into Tetrahymena cells and the biosynthesis of tetrahymanol is then completely inhibited (6, 11). It is also found that tetrahymanol was completely replaced by ergosterol in ciliary membranes (4).

Furthermore, ergosterol-supplemented cells indeed had fatty acid compositions different from those of the tetrahymanol-containing cells. Substitution of ergosterol led Tetrahymena to synthesize more short-chain fatty acids and less unsaturated fatty acids, as well as to increase the amount of an unusual isomer of linoleic acid, 6,11-18:2 (12, 5). It is particularly interesting that some of these differences can be traced to apparent shifts from the major pathway of fatty acid biosynthesis to the minor pathway (see below).

There are two pathways of fatty acid biosynthesis found in Tetrahymena (13, 14):

The first pathway is the main one in normal cells. The fatty acid products found in this pathway are: 9,12-18:2, representing 20% of the total fatty acids, and 6,9,12-18:3, 30% of the total (5).

The second pathway is a minor one in normal cells, but was much enhanced in ergosterol-supplemented cells. Ferguson et al (5) first identified the unusual isomer (6,11-18:2) of linoleic acid in the fatty acids of Tetrahymena. Koroly and Conner then proved the existence of

this minor pathway, by means of incubation of whole cells with radioactive precursors (14). Ferguson et al (5) found that the amount of 6,11-18:2 increased at the same time the amounts of 9,12-18:2 and 6,9,12-18:3 decreased when tetrahymanol was substituted by ergosterol in whole cells. The ratio of the amount of products (9,12-18:2 and 6,9,12-18:3) from the main pathway to the product (6,11-18:2) from the minor pathway in normal cells was 13.6:1 while this ratio was reduced to 10.6:1 (calculated from (5)) after ergosterol was supplemented. But Ferguson et al didn't find the mechanism by which ergosterol could influence either desaturation or elongation of fatty acids or their incorporation into phospholipids.

In the research of Maynard Neville (15), who was another worker in our laboratory, labelled precursor (^{14}C-9-18:1) was incubated with whole cells to investigate the presumed effect of ergosterol on the major pathway, and he found that the amounts of 9,12-18:2 and 6,9,12-18:3 produced were reduced after the supplement of ergosterol. In my research, substrate ^{14}C-cis-vaccenate was incubated with whole cells of both normal and ergosterol-supplemented cultures to investigate the presumed effect of ergosterol in the minor pathway. If the result shows that the production of 6,11-18:1 is significantly changed after the supplement of ergosterol, it then can be said that ergosterol seems to influence, in some way, the desaturation mechanism of fatty acid biosynthesis in Tetrahymena.

In principle, the phospholipid fatty acid composition (phospholipid is the main lipid in membrane) may be influenced:

1) by changes in the distribution of fatty acids biosynthesized de novo,

2) by selectivity at the level of incorporation of fatty acids into phospholipid as mediated by acyltransferases,

3) by alteration of the rates of turnover of fatty acyl groups of phospholipids in membranes, or

4) by a combination of these mechanisms. The results mentioned above (5, 15) revealed that mechanism one seems likely. This change would involve elongation (fatty acids from ergosterol-supplemented cells are comparatively shorter) and/or desaturation (the degree of unsaturation in ergosterol-supplemented cells is reduced).

The endoplasmic reticulum is an organelle of the cell where the desaturating and elongating enzyme systems are located (16). When the membranes of the endoplasmic reticulum are fragmented during breakage of cells, the membrane fragments are self-reassembled in the form of small, completely enclosed vesicles called microsomes (17).

It was found that ergosterol replaced tetrahymanol in Tetrahymena membranes (4), but the whole cell was able to contain 3-fold more ergosterol than tetrahymanol: Tetrahymena normal whole cells contained tetrahymanol in a molar ratio of 0.082 to phospholipids, while ergosterol-supplemented whole cells contained ergosterol in a molar ratio of 0.263 to phospholipids (calculated from (4)). More recent research of Ferguson (18) showed that the Tetrahymena microsomes were able to contain ergosterol 3.5-fold more than tetrahymanol: Tetrahymena normal microsomes contained tetrahymanol in a molar ratio of 0.04 to phospholipids, while ergosterol-supplemented microsomescontained ergosterol in a molar ratio of 0.14. This excessive amount of ergosterol might originate either as an artifact from food vacuoles broken during sonication, with simple

adsorption of ergosterol to the microsomes, or from actual incorporation into the microsomal membranes. The latter possibility was assumed in this research; the two alternatives cannot be distinguished at this time.

Microsomes were separated from both tetrahymanol-containing cells (normal cells) and ergosterol-supplemented cells. ^{14}C-cis-vaccenic acid, again, was used as the precursor to test the influence of ergosterol supplement on biosynthesis of fatty acids.

^{14}C-cis-vaccenic acid was the main precursor used in this research. Because it was commercially unavailable, it was made by growing Nitrobacter agilis with ^{14}C-acetate. Auran and Schmidt (19,20) showed that Nitrobacter contained cis-vaccenic acid in its lipids at up to 96% of total fatty acids, and that Nitrobacter was able to incorporate radioactive sodium acetate, which served as precursor for synthesizing fatty acids, without a change in fatty acid composition. The final cis-vaccenic acid preparation contained a trace of palmitoleic acid, which would not significantly interfere with this research, because it was itself a precursor of cis-vaccenic acid in Tetrahymena.

Materials and Methods:

A. Biosynthesis of Radioactive Cis-vaccenic Acid:

Cultures of Nitrobacter agilis (American Type Culture Collection) were grown under sterile conditions in inorganic medium containing per liter of water: $Na_2HPO_4 \cdot 7H_2O$, 3.3g; KH_2PO_4, 0.2g; $NaNO_2$, 1.38g; $MgSO_4 \cdot H_2O$, 10mg; $CaCl_2 \cdot 2H_2O$, 4mg; $CoCl_2 \cdot 6H_2O$, 20 µg; $ZnSO_4 \cdot 7H_2O$, 20 µg; $CuSO_4 \cdot 5H_2O$, 20 µg; $Na_2MoO_4 \cdot 2H_2O$, 20 µg; $FeSO_4$-EDTA solution, 10 ml. The last item was composed of 77 mg of $FeSO_4 \cdot 7H_2O$ plus 103 mg of disodium EDTA in 50 ml distilled water (19). The pH of the fresh medium was 7.8±0.2. Cultures were grown in a "shaking cart" at 20° ; the culture size was 400 ml in 1 liter Erlenmeyer flasks or 800 ml in 2 liter Erlenmeyer flasks, or 1 liter in 2.8 liter low-form Erlenmeyer flasks. The flasks were stopped by cheesecloth/cotton plugs. Cultures were inoculated from stock cultures grown in 20 to 200 ml medium at 20-30°. ^{14}C-sodium acetate (40-60 mCi/mmole, New England Nuclear Co.) was added to a concentration of 1 to 10 µM with 1 to 10 x 10^7 counts per minute to each culture.

The "shaking cart" was composed of a shaking water bath (Eberbach Co.), a laboratory cart and some elastic materials. The flasks were put in the cart, which was connected by rope to the shaking water bath. The elastic materials were sitting between the cart and shaking bath, and served as a buffer, which allowed the cart to be shaken by the water bath.

The Folch method (21) was used to extract the lipids from cells which had been removed from the medium by filtering through 0.45 micrometer millipore filters (Millipore Co.). The lower layer was

taken to dryness by an evaporation by N_2 stream.

During the extraction of lipids after filtering of cells, any filter fragments were removed; otherwise they would be dissolved together with lipids into the extracting solution and interfere with the following experiment.

Fatty acid methyl esters were prepared from extracted lipids by addition of 1-2 ml of a 0.5 N HCl/CH_3OH solution, flushing with N_2 and heating in a heating block (Lab-Line Inc.) at about 75°C for 1-2 hours. One milliliter of distilled water was then added and the methyl esters were extracted three times into a total of six milliliters of petroleum ether. Solvent was removed by evaporation under a N_2 stream.

Argentation chromatography was used to separate the fatty methyl esters according to chain length and number of double bonds. Methyl esters were applied as a solution in 1 milliliter petroleum ether to a 0.5 g silver nitrate-impregnated Unisil (20% $AgNO_3$ w/w) column. The column was eluted with increasing proportions of benzene in petroleum ether followed by pure diethyl ether, required to remove all of the 11-18:1 from the columns. The system is described below and the separated methyl esters contained in each eluate listed, as determined by gas-liquid chromatography using a Varian Aerograph #286010-00 (Varian Corp.) with a four foot glass column containing 15% HI-EFF-8P (DGS) on Chromosorb P (80-100 mesh) and operation column temperature of 160°C. Methyl cis-vaccenate of known concentration, which was made from commercial cis-vaccenic acid (Analabs, Inc.), served as the standard. The calculation of relative retention time based on a methyl stearate standard allowed identification of the unknown esters

in eluates, and the peak area ratio of the unknown to the standard allowed the calculation of the unknown concentrations in eluates. Many other column systems were investigated during the course of the work (data not shown) to find the optimum system yielding the necessary separation. The system shown below allows for separation of pure 11-18:1.

	Amount of solvent	Composition of solvent	Methyl esters identified in eluate
1)	20 ml	15% Benzene in petroleum ether (v/v)	16:0
2)	20 ml	50% Benzene in petroleum ether (v/v)	18:1, 16:1 (trace)
3)	10 ml	Diethyl ether	18:1 (trace), 16:1 (trace)

The second eluate, which contained radioactive methyl cis-vaccenate as the major constituent, was hydrolyzed in 1-2 milliliters 10% KOH in CH_3OH/H_2O (8:2 v/v) in a heating block at 37°C for about 2 hours, shaking occasionally. One milliliter of distilled water was added and the mixture was acidified by addition of an excess of concentrated hydrochloric acid. Free fatty acid was extracted three times into a total of six milliliters of petroleum ether. One tenth ml of this solution was placed in a scintillation vial to measure radioactivity. Five milliliters toluene-base scintillation fluid (6.0 gm POP plus 0.1 gm POPOP (Eastman) in 1 liter scintillation grade toluene (Fisher)) were added and the sample was counted for two minutes in a Beckman LS-100C liquid scintillation counter. A background count was taken prior to counting of experimental samples by doing the same procedures but with scintillation fluid only. The rest of the radio-

active cis-vaccenic acid in the petroleum ether solution was dried by N_2 stream and redissolved in a small amount of absolute ethanol and stored in a freezer.

Most of the substrate used with Tetrahymena in this research was the pure radioactive cis-vaccenic acid as isolated from Nitrobacter; however, in the experiment on effect of concentration of substrate, it was used in a mixture with nonradioactive cis-vaccenic acid (Analabs, Inc.) to give concentrations of 0.1 µg, 655 µg, 1964 µg, 2620 µg and 7200 µg per milliliter of absolute alcohol. The substrate used in every sample had a constant activity of about 10^5 cpm, but variable cis-vaccenate concentration.

B. Effect of Ergosterol on Fatty Acid Biosynthesis of Tetrahymena

Cultures of Tetrahymena pyriformis W were grown under sterile conditions in proteose peptone medium (2% proteose peptone, 0.1% yeast extract, 90 micromolar Fe^{+3}-EDTA complex, and distilled water to a total volume of 500 ml). Culture size was 20 ml in 50 ml Erlenmeyer cheesecloth/cotton stoppered flasks. Growth temperature was maintained at either 20^{o} or 29.5^{o}. Cultures were inoculated from stock cultures grown in 2.5 ml medium at either 20^{o} or 29.5^{o}, in such amount, i.e. 0.4 ml per culture, as to insure that the later incubation with substrate would take place at the time the culture was in log growth phase. Cultures were maintained in a constant temperature incubator (Forma Scientific) for either 16 hours at 29.5^{o} or 21 hours at 20^{o}.

Cultures were divided into two sets: one set was ergosterol cultures and the other set was tetrahymanol cultures. Small amounts of ergosterol solution (5mg/ml in ethanol) were added into each ergosterol

culture, while an equivalent amount of absolute ethanol was added into each tetrahymanol culture.

There are many variables which will influence the results. These are the amount and exposure time to ergosterol as well as the amount and incubation time with substrate (^{14}C-cis-vaccenic acid): ergosterol solution was added in different amounts (0.01, 0.02, 0.03, 0.04, and 0.05 ml) and for different times (1, 2, 3, and 4 hours) to determine the optimum amount and exposure time for ergosterol. As will be shown in Results, the earlier experiments with ergosterol exposure times from 1 to 4 hours didn't give consistent results. Ergosterol cultures were then exposed to ergosterol from the beginning of cell growth: that is, cells and ergosterol were added to the culture medium simultaneously. Substrate (radioactive cis-vaccenic acid) was added at different concentrations per culture (10^{-2} μg, 65.5 μg, 196.4 μg, 262.0 μg, and 720.0 μg) and incubated for different periods (0.5 hr, 1 hr, 1.5 hrs, and 2 hrs) to determine the optimum concentration and incubation time for substrate.

The methods of filtering Tetrahymena cells, extraction of lipids and esterification of fatty acids were done by the same method and materials as those done to Nitrobacter shown in Part A, except that the pore size of filters for Tetrahymena cells was 3 micrometers.

Argentation chromatography (see Part A) was used again to separate the Tetrahymena fatty acid methyl esters, but the elution pattern was changed as follows:

	Amount of solvent	Composition of solvent	Methyl esters identified in eluates
1)	20 ml	40% Benzene/Petroleum ether (v/v)	X:0, X:1, 11-18:1

2)	10 ml	75% Benzene/Petroleum ether (v/v)	11:18:1 (trace) 9,12-18:2
3)	15 ml	95% Benzene/Diethyl ether (v/v)	9,12-18:2 (trace) 6,11-18:2
4)	10 ml	Diethylether	6,11-18:2 (trace) 6,9,12-18:3

The esters in each eluate were identified again by gas-liquid chromatography, as described in Part A.

The above separated eluates were placed into scintillation vials and air-dried. Scintillation fluid was added and the samples were counted as described in Part A.

In every set of argentation chromatography procedures, one background column was prepared for correction for background contamination. This column had the equivalent amount of Tetrahymena esters and equivalent amount of ester of ^{14}C-cis-vaccenic acid. The radioactivity of each fraction was calculated as the percentage of overall counts from the background column, then subtracted from the percentage of the corresponding fraction of the sample columns as a correction for substrate contamination. The column procedure does give some trailing and imprecise separation.

The difference of percentages in the third fraction (6,11-18:2) between tetrahymanol and ergosterol cultures represented the difference of production of 6,11-18:2 between tetrahymanol cells and ergosterol cells.

Ergosterol (mp 162-164^{o}C, Sigma Chemical Co.) was recrystallized from methanol. Unisil (100-200 mesh, Clarkson Chemical Co.) was washed with hot methanol. The prepared $AgNO_3$-Unisil , which was made by

mixing $AgNO_3$ with washed Unisil (1:4 w/w) adding water to mix and then removing water by rotary evaporation, was stored in the dark at 110°C to avoid photo-decomposition and hydration. Columns were shielded with aluminum foil to prevent photodecomposition during chromatography.

Results:

It is apparent that whole cell studies are necessary before microsomal preparations would be useful (15). Some preliminary work was done (data not shown) which showed that cis-vaccenate was a better overall substrate than oleate for desaturation by Tetrahymena. Cis-vaccenate may be used to examine the activity of a proposed 6-desaturase which carries out the last reaction of the minor pathway (14): 16:0 → 9-16:1 → 11-18:1 → 6,11-18:2. A column technique was developed to isolate 6,11-18:2 from the substrate so that product formation could be measured (see Methods).

Table I shows the effect of substrate concentration on enzyme activity at either 20^{0} or 29.5^{0}. Both ergosterol supplemented and nonsupplemented cultures were investigated. Ergosterol solution (5 mg/ml ethanol) was added to 20 ml cultures of Tetrahymena at the late log growth phase. After three hours, combinations of ^{14}C-labeled 11-18:1 and nonlabeled 11-18:1 mixed to give various concentrations of the substrate were incubated with cultures for one hour. Results at both 20^{0} and 29.5^{0} show that higher concentrations of cis-vaccenate do not enhance the difference between the enzyme activities of normal and ergosterol supplemented cells to any significant extent; however, at very low concentrations (0.02 µg ^{14}C-cis-vaccenate only, no carrier added) there was a significant difference in enzyme activity. For this reason subsequent experiments were carried out using only labeled cis-vaccenate as substrate.

The effect of incubation time with substrate on enzyme activity also was investigated at 20^{0} and 29.5^{0}. The culture sizes, the conditions

of growth, the addition of ergosterol, and the addition of substrate were all the same as described above, but the incubation time with substrate was varied from 0.5 to 2.0 hours. Table II shows that the optimum time for substrate incubation at 20^{o} was between one and two hours, while the best time at 29.5^{o} was one hour; however, for ease of comparison with other workers' data (15), a one hour incubation time was used in subsequent experiments.

Initial experiments to investigate the effect of ergosterol supplement on enzyme activity were performed using twenty ml cultures, either ergosterol supplemented or nonsupplemented, grown at 20^{o} or 29.5^{o}. Table III lists the results of experiments to find the optimum concentration of ergosterol needed to show a difference in 6-desaturation. The final concentrations of ergosterol used were 0.15, 0.20, 0.25, 0.30 and 0.35 mg/20 ml culture. Cells were grown either with or without the sterol supplement prior to incubation with substrate. Percentages indicate the percent in the product fraction of the total counts per minute recovered from column chromatography following extraction of lipids by the modified Folch partition method and methylation by HCl/CH_3OH described in Methods. The results showed that 0.20 and 0.25 mg of ergosterol per culture produce the best effect of ergosterol on 6-desaturase activity. Since 0.20 mg/culture (0.01 mg/ml) is the same ergosterol concentration as used in earlier studies (5, 16), this concentration was used in subsequent experiments for ease in comparison of data.

Table IV lists the results of an experiment designed to find the optimum exposure time of Tetrahymena to ergosterol at 20^{o} and 29.5^{o}. The experiments were done under the same conditions as those described

in Table III, except that the growth in the presence of ergosterol prior to addition of substrate varied from zero to four hours. Both results at 20^{o} and 29.5^{o} showed that the best difference in 6-desaturase enzyme activity is observed after three hours exposure of the cells to ergosterol; however, another experiment was done to lengthen the exposure of cells to ergosterol to much longer time by adding ergosterol at the start of growth of cells, and the result is shown in Table V. These experimental conditions,at both 20^{o} and 29.5^{o}, show the best results of this research. The effect of the long-term ergosterol supplement is to cause Tetrahymena pyriformis W cells to synthesize an average of seventeen percent more 6,11-18:2 than normal cells when incubated with ^{14}C-cis-vaccenate for one hour. In all experiments, desaturation of ^{14}C-11-18:1 was generally higher in ergosterol-supplemented cells, but the data in Table V are much more convincing than earlier experimental results.

Discussion:

This research has been devoted to finding the optimum conditions for enzyme activity of a proposed 6-desaturase (see Tables I, II), the best dose of ergosterol and its best exposure time (see Tables III,IV). Although all the results listed in these four Tables were invalid when judged by the statistical significance (the number of experiments are not sufficient), they seemed to show a positive ergosterol effect. However, the final experiment with long-term exposure of ergosterol (see Table V) gave the best result and was statistically significant after applying Student's t-test (the motive of this experiment is from the results of M. E. Neville (15), who found that long-term exposure to ergosterol did have a definite influence on the desaturation in the major pathway of Tetrahymena).

The results shown in Table V suggest that supplement of ergosterol in Tetrahymena at the whole cell level leads to an increased capacity to introduce a double bond at C-6 in the minor pathway. Two interpretations of this result may be proposed, and lead to directions to perform further investigations.

There must be at least one enzyme system involved in the desaturation of cis-vaccenate. The desaturases of eucaryotic organisms are located in the endoplasmic reticulum (22). Ergosterol might affect fluidity properties of the phospholipids of both the endoplasmic reticulum in vivo and the microsomes in vitro (18). Alternatively, this desaturase system might be genetically controlled to respond to sterol substitution (18). One preliminary experiment some time ago done by me showed

that microsomal fatty acid composition seems to be altered. The microsomes from ergosterol grown cells had significantly more 6,11-18:2 than normal cell microsomes (data not shown). The most recent research (18) does show the same alteration. These results also show that ergosterol seems to enhance the desaturation capacity at C-6 in the minor pathway. Another investigation done by M. E. Neville shows that ergosterol seems to reduce the capacity of Tetrahymena to introduce a double bond at C-12 in the major pathway (15, 18). Both of the above results show the significant effect of ergosterol on desaturation.

The direct effect of ergosterol substitution might be at the level of acylation of precursors or of phospholipase-catalyzed turnover of membrane-bound acyl groups, or of some more general phenomena, such as the rate of transport or uptake of the substrates into the microsomes (18). Further investigations might resolve these multiple possibilities. For example, the last possibility can be studied if an investigation is done to compare the rate of uptake of the cis-vaccenate into the microsomes, from both ergosterol grown and normal cells under the optimum desaturation conditions established in this research (18). No matter what the mechanism of the ergosterol effect is proved to be, the observation of higher levels of 6,11-18:2 may be important to connect with the observation of lower levels of 9,12-18:2 in ergosterol-containing cells. This may be a "feedback" phenomenon in Tetrahymena: it is proposed that Tetrahymena cells synthesize more 6,11-18:2 in the minor pathway and less 9,12-18:2 in the major pathway in order to maintain their original optimum membrane fluidity when ergosterol is incor-

porated into the membrane. These Tetrahymena may incorporate the foreign sterol into their own membranes, saving the energy required to synthesize their native sterol, requiring a "feedback" regulation. It seems that such a "feedback" phenomenon would be of use in any sterol-containing organism, including humans, to regulate the production of fatty acids suitable for maintaining the original optimum membrane fluidity and might also provide a partial explanation for the mechanism of some human diseases, familial hypercholesterolemia and atherosclerosis, caused by excess of cholesterol.

TABLE I

THE EFFECT OF SUBSTRATE CONCENTRATION ON THE FORMATION OF LABELED 6,11-18:2[a] FOR NORMAL(TOL) AND ERGOSTEROL-SUPPLEMENTED CELLS(EOL)[b] GROWN AT 20° AND 29.5°.

SUBSTRATE ADDED TO 20 ml CULTURE[c]	%6,11-18:2 PRODUCED[d] TOL	EOL	DIFFERENCE (EOL - TOL)
	20°		
0.01 µg	30 ± 4	43 ± 4	+13
66 µg	10 ± 1	11 ± 1	+1
196 µg	6 ± 1	13 ± 2	+7
262 µg	10 ± 2	3 ± 1	-7
720 µg	8 ± 2	5 ± 1	-3
	29.5°		
0.01 µg	13 ± 2	20 ± 3	+7
66 µg	7 ± 1	9 ± 1	+2
196 µg	4 ± 1	7 ± 1	+3
262 µg	7 ± 1	5 ± 1	-2
720 µg	0	2 ± 2	+2

a. Values are expressed as the percent of total counts per minute recovered following a one hour incubation with ^{14}C-labeled 11-18:1, extraction of lipids by the modified method of Folch and methylation with HCl/CH_3OH (see Methods).

b. Cells were grown in 20 ml medium supplemented with ergosterol or an equivalent amount of ethanol at the time of late log growth phase of the culture (see Methods).

c. Every concentration except the first one (0.01 µg/culture) was a combination of ^{14}C-cis-vaccenate and nonlabeled vaccenate, while the first one was the pure ^{14}C-cis-vaccenate only.

d. Each value is the average of three experiments at 20° or two experiments at 29.5° The standard deviation was calculated using the formula $S = [(x_1 - m)^2 + \cdots + (x_n - m)^2]^{1/2}/(n - 1)^{1/2}$.

TABLE II

DESATURATION OF CIS- VACCENATE BY *TETRAHYMENA* AS A FUNCTION OF TIME OF EXPOSURE OF CELLS TO THE ^{14}C-LABELED SUBSTRATE

SUBSTRATE EXPOSURE TIME[a]	% 6,11-18:2 FORMED[b] TOL	EOL	DIFFERENCE (EOL - TOL)
	20°		
0.5 hr	23 ± 1	18 ± 2	-5
1.0 hr	22 ± 0	28 ± 2	+6
1.5 hrs	30 ± 2	34 ± 2	+4
1.75 hrs	32 ± 2	35 ± 1	+3
2.0 hrs	29 ± 3	39 ± 3	+10
	29.5°		
0.25 hr	7 ± 1	7 ± 1	0
1.0 hr	11 ± 2	15 ± 2	+4
1.25 hrs	12 ± 2	15 ± 2	+3
2.0 hrs	17 ± 1	18 ± 2	+1

a. The concentration of substrate used in 20° experiments was 0.02 µg/culture, but in 29.5° experiments was 150 µg/culture. Ergosterol solution (0.2 mg/culture) or an equivalent amount of ethanol was added three hours before the addition of substrate. Prior to the addition of ergosterol cultures had grown for 16 h (20°) or 12 h (29.5°).

b. Values are expressed as the percent of total counts per minute recovered (see Methods), and are the average of two experiments.

TABLE III

THE EFFECT OF ERGOSTEROL CONCENTRATION ON THE FORMATION OF LABELED 6,11-18:2 FOR NORMAL(TOL) AND ERGOSTEROL-SUPPLEMENTED CELLS(EOL) GROWN AT 29.5°

ERGOSTEROL CONCENTRATION[a]	% 6,11-18:2 FORMED[b] TOL	EOL	DIFFERENCE (EOL - TOL)
0.15 mg/culture	7.0	8.5	+1.5
0.20 mg/culture	7.7	9.7	+2.0
0.25 mg/culture	5.0	7.6	+2.6
0.30 mg/culture	4.0	5.6	+1.6
0.35 mg/culture	5.9	4.1	-1.8

a. Ergosterol solution was added at the late-log growth phase. The substrate (11-18:1) was added three hours later, and incubated with cells for one hour (see Methods).

b. Values are expressed as the percent of total counts per minute recovered (see Methods), and represent the average of two experiments.

TABLE IV

THE EFFECT OF TIME GROWTH WITH ERGOSTEROL ON THE FORMATION OF LABELED 6,11-18:2 FOR NORMAL(TOL) AND ERGOSTEROL-SUPPLEMENTED CELLS(EOL) GROWN AT 20° AND 29.5°

GROWTH WITH ERGOSTEROL[a]	% 6,11-18:2 FORMED[b] TOL	EOL	DIFFERENCE (EOL - TOL)
	20°		
0.25 h	24	28	+4
1.0 h	22	23	+1
2.0 h	28	24	-4
3.0 h	22	27	+5
4.0 h	23	25	+2
	29.5°		
0 h	13	13	0
1.0 h	18	14	-4
2.0 h	18	23	+5
3.0 h	24	34	+10
4.0 h	19	19	0

a. Ergosterol was added in the late log growth phase; after growth for the time indicated, substrate was added and incubated for one hour and then the cultures were harvested (see Methods).

b. Values are expressed as the percent of total counts per minute recovered (see Methods), from one experiment only.

TABLE V

THE EFFECT OF LONG-TERM GROWTH WITH ERGOSTEROL AT 20° AND 29.5° ON THE FORMATION OF LABELED 6,11-18:2 FOR NORMAL (TOL) AND ERGOSTEROL-SUPPLEMENTED CELLS(EOL)

EXPERIMENT[a]	% 6,11-18:2 FORMED[b]		DIFFERENCE
	TOL	EOL	(EOL - TOL)
	20°		
1	35	52	+17
2	34	52	+18
3	26	45	+19
4	31	48	+17
Average	31 ± 4	49 ± 3	+18
	29.5°		
1	25	43	+18
2	24	39	+15
Average	25 ± 1	41 ± 2	+16

a. Cells were grown for 14 h at 29.5° or 24 h at 20° in the medium supplemented with ergosterol or nonsupplemented. ^{14}C-Cis-18:1 was then added and incubated for one hour, and extraction of lipids was done by the modified method of Folch (see Methods).

b. Values are expressed as the percent of total counts per minute recovered (see Methods).

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