Metabolism of cyclopropane fatty acids by Tetrahymena pyriformis

Najah M. Al-Shathir
Iowa State University
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Metabolism of cyclopropane fatty acids by

*Tetrahymena pyriformis*

by

Najah M. Al-Shathir

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INTRODUCTION

Cyclopropane fatty acids are known to occur in a wide variety of bacteria (Kates, 1964) and in some plants (Hooper and Law, 1965; Johnson et al., 1967; Kleiman et al., 1968; Yano et al., 1972a; Kuiper and Stuiver, 1972) and protozoa (Meyer and Holz, 1966). They have been also detected in the millipede Graphidostreptus tumuliporus (Oudejans et al., 1971; Van der Horst et al., 1972) and in sheep rumen tissue (Brody, 1972).

Higher animals have been shown to degrade cyclopropane fatty acids to the ring, leading to the accumulation of cyclopropane fatty acids with shorter chains (Wood and Reiser, 1965; Chung, 1966). No accumulation of such short-chain cyclopropane fatty acids was reported in protozoa feeding on bacterial cells rich in these acids.

Ochromonas danica, a photosynthetic photophlagellate protozoan capable of ingesting bacteria, was reported to be able to oxidize the methylene carbon of cyclopropane fatty acids to CO₂ (Magat and Tipton, 1970). In an extension of this work, this organism was used to study the biodegradation of the cyclopropane fatty acids that are naturally present in Escherichia coli cells. When E. coli cells were fed to this protozoan, no cyclopropane fatty acids biodegradation could be detected by gas-liquid chromatography.

The free living ciliate, Tetrahymena pyriformis was then
chosen to study the biodegradation of cyclopropane fatty acids in vivo and in vitro. This organism was chosen because it is easy to grow on a defined medium as well as on a bacterial culture. It is capable of ingesting all E. coli cells on a culture medium in a few days. Moreover, it was reported to be able to take and utilize exogenous long-chain fatty acids from the medium (Lees and Korn, 1966).

The objectives of this work are to study the fate of cyclopropane fatty acids in nature, to detect the products of their oxidation, and to test a mechanism for their oxidation.
REVIEW OF LITERATURE

Cyclopropane Fatty Acids

Occurrence

In 1950, Hofmann and Lucas isolated from the bacterium Lactobacillus arabinosus a C_{19} fatty acid (lactobacillic acid) that contained a cyclopropane ring. The structure was demonstrated to be cis-11,12-methyleneoctadecanoic acid (Hofmann, 1963). The occurrence of a C_{17} cyclopropane fatty acid (CFA) in Escherichia coli was then reported (O'Leary, 1959a; Dauchy and Asselineau, 1960) and it was proved to be cis-9,10-methylenehexadecanoic acid by Kaneshiro and Marr (1961). Lactobacillic acid and an isomer cis-9,10-methyleneoctadecanoic acid or dihydrosterculic acid occur in Salmonella typhimurium (Gray, 1962). Dihydrosterculic acid is also the principal CFA in several flagellate protozoa (Meyer and Holz, 1966). Several cyclopropane fatty acids (CFAs) of different chain lengths have been shown by gas liquid chromatography (GLC) to occur in nature, for example, C_{13} and C_{15} CFAs in Clostridia butyricum (Goldfine and Bloch, 1961) and in Pleuropneumonia-like organisms (O'Leary, 1962), and a C_{21} CFA in Rhodomicrobium vannielii (Park and Berger, 1967).

The fatty acid composition of any bacterial species can vary considerably with the conditions under which it is grown (Kates, 1964). The age of the culture, the concentration of certain ions and nutrients, pH and temperature can all affect
the amount of CFAs synthesized. An increase in the proportion of CFAs in bacterial cultures at the stationary phase of growth has been reported to occur in E. coli (Marr and Ingraham, 1962; Law et al., 1963; Knivett and Cullen, 1965), Serratia marcescens (Law et al., 1963; Kates et al., 1964) and Agrobacterium tumefaciens (Law et al., 1963). There is a decrease in CFAs in E. coli grown in alkaline media, in the presence of citrate or in low concentrations of Mg$^{2+}$ or SO$_4^{2-}$ (Knivett and Cullen, 1965; 1967). When E. coli is grown over a temperature range of 43-10°C, the proportion of CFAs decreases to a low value at the low temperature (Marr and Ingraham, 1962).

CFAs are present not only in bacteria and protozoa, but also in some plants. Dihydrosterculic acid constitutes 17.4% of the seed oil of Dimocarpus longans, Sapindaceae (Kleiman et al., 1968). CFAs are also reported in the seedlings of species of the order Malvales (Hooper and Law, 1965; Johnson et al., 1967) and in small amounts in all other tissues of the same plants (Yano et al., 1972a). Long chain CFAs with 25 carbon atoms were observed recently by Kuiper and Stuiver (1972) in the leaves of the early spring plants snow drop (Galanthus nivalis L.), cow parsley (Anthriscus silvestris L.), rye (Secale cereale L.), English ryegrass (Lolium perenne L.) and wheat (Triticum estivum L.). Several other CFAs (C$_{21}$, C$_{22}$, C$_{23}$ and C$_{25}$) were also reported by the same authors to occur in the drought-tolerant Corynebacterium canescens.
Beside microorganisms and plants, several CFAs (C_{17}, C_{18}, and C_{19}) have been isolated recently from the millipede *Graphidostreptus tumuliporus* (Oudejans et al., 1971) where they are present in the females and eggs but not in the males (Van der Horst et al., 1972).

Recently, two unusual CFAs, 2,3-methylenehexadecanoic and 2,3-methylenoctadecanoic acids have been isolated from sheep rumen tissue by Brody (1972). The presence of CFAs in rumen tissue is not unexpected. Of 21 pure cultures of rumen bacteria representing 12 genera and 14 species, Ifkovits and Ragheb (1968) noticed the presence of C_{19} CFA(s) in 4 of them at a range of 1.6%-14% of the total fatty acid content. However, the structure of the CFAs in rumen tissue reported by Brody (1972) is a complete surprise.

**Biological carrier**

In bacteria and protozoa, CFAs have been found only as constituents of phospholipids. However, Kito et al. (1972) recently reported the presence of free *cis*-9,10-methylenehexadecanoic acid in *E. coli*, and there was a ten-fold increase in the amount of that acid between the early exponential and the stationary phase of growth. All polar lipid classes of *E. coli* (Kanemasa et al., 1967), *A. tumefaciens* (Hildebrand and Law, 1964) and *L. casei* (Thorne, 1964) contain similar proportions of CFAs. In protozoa the CFAs are exclusively esterified to phosphatidylethanolamine (Meyer and Holz, 1966).
The distribution of fatty acids in purified phospholipids isolated from several different bacteria has been examined by Hildebrand and Law (1964). Phosphatidylethanolamine from E. coli, S. marcescens and A. tumefaciens and phosphatidylcholine from A. tumefaciens contain the CFAs esterified predominantly to the 2- position. A notable exception to this general distribution rule has been encountered with the phosphatidylethanolamine of Clostridium butyricum in which the CFAs are found more abundantly at the 1- position. In Brucella abortus Bang, although CFAs are located preferentially at the 2-position of phosphatides (Thiele et al., 1968), some of them are present in appreciable amounts at the 1- position, especially in phosphatidylcholine and cardiolipin. More detailed examination of the extracellular phosphatidylethanolamine isolated from a lipopolysaccharide-phospholipid-protein complex produced by E. coli has shown that the major individual molecular species containing all the CFAs is 1-palmitoyl-2-cis-9,10-methylenehexadecanoyl-sn-glycerol-3-phosphoryl-ethanolamine (Van Golde and Van Deenen, 1967).

In higher plants of the order Malvales the CFAs are mainly concentrated in the neutral lipids with a lesser proportion in phospholipid or glycolipid classes (Yano et al., 1972a). Comparison of the fatty acid composition of the glyco-, phoso-, and sulfolipids of the early spring plants snow drop and rye (Kuiper and Stuiver, 1972) showed that CFAs were only found in the sulfolipid fractions of both species,
and a small amount in the phosphatidylinositol fraction of snow drop. The same author also observed that the drouth-tolerant species C. canescens contains CPAs only in the phosphatidylcholine fraction. In the millipede G. tumuliporus, the CPAs are present not only in phospholipid fraction, but also in an even higher amount in the neutral lipids (Van der Horst et al., 1972). The CPAs of the sheep rumen tissue (Brody, 1972) are located only in phosphatidylethanolamine and are esterified to the 1-position of this phospholipid.

**Biosynthesis**

Using labelled vaccenic and oleic acids, O'Leary (1959 a, b) demonstrated that in *E. coli* and *L. arabinosus* those unsaturated fatty acids are incorporated intact into lactobacillic acid and that the methyl carbon from exogenous methionine-methyl-\(^{14}\)C serves as the source of the methylene carbon. This was soon confirmed to occur in *L. arabinosus* (Hofmann and Liu, 1960) and in *E. coli* and *L. casei* (Chalk and Kodicek, 1961). Furthermore, the results from the addition of vaccenic acid and [methyl-\(^{14}\)C] methionine or \(^{14}\)C-formate to the medium of *L. arabinosus* (Liu and Hofmann, 1962) and chemical degradation of the labelled lactobacillic acid formed, demonstrate conclusively that lactobacillic acid synthesis involves the addition of a one-carbon fragment across the double bond of cis-vaccenic acid. By the use of auxotrophic mutants of *Aerobacter aerogenes* which have varying requirements
for exogenous sources of methionine and S-adenosylmethionine (O'Leary, 1962), or extracts of either S. marescens or C. butyricum (Zalkin et al., 1963), it has been shown that S-adenosylmethionine is the actual donor of the one carbon unit. Two of the hydrogen atoms of the methyl group are incorporated into the cyclopropane ring (Pohl et al., 1963), and both hydrogen atoms of the double bond of oleic acid are retained during its conversion to dihydrosterculic acid by L. arabinosus (Polachek et al., 1966). This finding ruled out intermediates in cyclopropane ring formation which have double bonds at carbon atom 9 or 10 after the extra carbon has been added, e.g., a 9,10-cyclopropene compound, 9- or 10-methylene compound, or 9,10-olefins with a branched methyl group at position 9 or 10.

A similar process for the biosynthesis of CFAs seems to take place in protozoa (Meyer and Holz, 1966), Hibiscus seedlings (Hooper and Law, 1965) and in the Malvaceae (Johnson et al., 1967; Yano et al., 1972b).

A crude enzyme from C. butyricum is inactive in CFA synthesis unless an aqueous dispersion of phospholipids containing unsaturated fatty acids is added (Zalkin et al., 1963). However, the same authors found that no activation of synthesis by an aqueous dispersion of phospholipid occurs with crude extract from S. marescens. Extracts from both organisms contain endogenous phospholipids and the product of the reaction isolated from incubation mixtures of either enzyme source is
primarily phosphatidylethanolamine which contains CFAs. Use of a more purified enzyme preparation from \textit{L. arabinosus} showed that phosphatidylethanolamine is the real substrate for the enzyme cyclopropane synthetase (Chung and Law, 1964).

As was mentioned before, CFAs are generally located at the 2-position of phospholipids of bacteria with the exception of \textit{C. butyricum} where they are located predominantly at the 1-position. The specificity of the \textit{C. butyricum} cyclopropane synthetase reaction has been examined by employing as substrate a phosphatidylethanolamine with a known distribution of unsaturated fatty acids and analyzing the distribution of CFAs in the phospholipid produced by the enzyme reaction (Hildebrand and Law, 1964). These experiments indicate that the enzyme has a definite, but not absolute, specificity for an unsaturated fatty acid at the 1-position. On incubation of a purified plasmalogen with crude and purified CPA synthetase from \textit{C. butyricum}, incorporation of the methyl group of \textit{[Methyl-^{14}C]} S-adenosylmethionine into lipid was observed (Chung and Goldfine, 1965). Sixty percent of the \textit{^{14}C} was found in the aldehyde and 40\% in the fatty acid chains. Also, several other natural and synthetic phospholipids can serve as substrates for the CPA synthetase of \textit{C. butyricum} (Thomas and Law, 1966). A diether analogue of phosphatidylethanolamine, 1,2-di-(9-octadecenoyloxy)-3-(2-aminoethyl) phosphorylpropane, is an effective substrate for the CPA synthetase. Phosphatidic acid, phosphatidylglycerol and phosphatidylserine can also
serve as substrates for the enzyme. These results indicate that the ester carbonyl groups are not essential for the enzyme activity and that phospholipids are the natural substrates for the CFA synthetase.

**Biodegradation**

Very little is known about the CFAs degradation. Feeding rats, placed on a fat-free diet, racemic methyl cis- and trans-9,10-methyleneoctadecanoic acids led to the accumulation of cis- and trans-3,4 methylenedodecanoic acids in their adipose tissue (Wood and Reiser, 1965). Normal fatty acid metabolism appeared unaffected by the low level of the CFAs fed. Chung (1966) found that when cis-9,10-methylenehexadecanoic and cis-9,10-methyleneoctadecanoic acids were incubated with rat liver mitochondria, the mitochondria converted these long chain fatty acids to shorter chain CFAs (cis-3,4-methylenedecanoic and cis-3,4-methylenedodecanoic acids, respectively). Also, the methylene carbon of the cyclopropane ring of (9,10-methylene$^{14}$C)-cis-9,10-methylenehexadecanoic acid was not converted to $^{14}$CO$_2$. It seems that the cis- and trans-CFAs isomers can be equally well metabolized by the rat, but the acids are catabolized, presumably by beta oxidation, only down to the ring where the enzyme system is apparently unable to degrade the rest of the chain.

Only two examples are given in the literature that show complete biodegradation of CFAs. The first one is an in vivo
experiment performed by Magat and Tipton (1970). In their experiment [9,10-methylene-$^{14}$C]-cis-9,10-methylenehexadecanoate and [11,12-methylene-$^{14}$C]-cis-11,12-methyleneoctadecanoate were fed to the protozoan, Ochromonas danica and a significant label appeared as CO$_2$. The second example was reported by Schiller (1970). When he incubated A. tumefaciens cells in the presence of [9,10-methylene-$^{14}$C] cis-9,10-methylenehexadecanoic acid, $^{14}$CO$_2$ was also released from the incubation medium.

**Physiological role**

A lot of work is still to be done to understand the role of these acids in the organisms containing them. They are not present in all bacteria and those containing them show different degrees of accumulation depending on the age of their culture, pH, temperature and the concentration of certain ions and nutrients (Kates, 1964). Such acids are also reported only in certain plants (Hooper and Law, 1965; Johnson et al., 1967); in some of these plants they are present in all tissues, but at the highest concentration in immature seeds (Yano et al., 1972a). Some early spring plants containing them show none of these acids when collected later in the spring and species of the drought-tolerant plants show a reduction in these acids after a rainy period (Kuiper and Stuiver, 1972). In the millipede G. tumuliporus, CPAs account for 25% of total fatty acids in the female (without eggs), and for 35% in the eggs, while in the male the presence of these fatty acids could not
be detected (Van der Horst, 1972).

Some of the earlier studies of the metabolic activity of these acids showed that a number of long chain CFAs, both naturally occurring and synthetic, have the ability to substitute for biotin in the nutrition of \textit{L. arabinosus}, \textit{L. casei} and \textit{L. delbrueckii} (Hofmann and Panos, 1954). Furthermore, \textit{L. arabinosus} and \textit{L. casei} failed to produce measurable amounts of \textit{cis}-vaccenic acid when lactobacillic acid replaced biotin in their cultural medium (Hofmann et al., 1959). In other words, lactobacillic acid is capable of substituting metabolically for \textit{cis}-vaccenic acid. The cyclopropane analogues of oleate, linoleate and arachidonate fed to essential fatty acid-deficient rats for 8 days induced no change, compared to essential fatty acid deficient rats, in the microsomal or mitochondrial fatty acid pattern of the liver (Guarnieri and Johnson, 1970). Also, the CFAs could not be detected in microsomes or mitochondria by GLC or infrared analysis. Although there was some accumulation of those acids in the adipose tissue, CFAs induced no change in the fatty acid pattern characteristic of essential fatty acid deficiency. It seems that CFAs derived from linoleate and arachidonate have no essential fatty acid activity, although a longer time of feeding them is probably required. On the other hand, the inability of rat phospholipids to accumulate the CFAs derived from linoleate and arachidonate in their phospholipids may be explained by the finding that the acyl transfer rate of oleic
acid to 1-acyl-sn-glycerol-3-phosphorylcholine is about 10 times greater than that of 9,10-methyleneoctadecanoate (Okuyama et al., 1969). The same authors found that there was selective transfer of CFAs by acyl coenzyme A : phospholipid acyltransferase from rat liver microsomes. The thiol esters of the naturally occurring CFAs, **cis-9,10-** and **cis-11,12-** methyleneoctadecanoic acids, are substrates for acyl transferases that form phospholipids. Therefore, it is conceivable that these acids could be incorporated into the cell membrane structure of animal tissue. In **E. coli**, acyl coenzyme A : L. glycerol acyltransferase, which is a particulate enzyme, was found to be inhibited by **cis-9,10-methylenehexadecanoate** (Kito et al., 1972). Palmitate, elaidate and **trans-vaccenate** showed no inhibition, whereas palmitoleate, oleate and **cis-vaccenate** inhibited. **Cis-9,10-methylenehexadecanoic acid** was twice as inhibitory as the mono-olefinic acids. Henderson and McNiel (1966) found that when dihydrosterculic acid was added to **L. plantarum** in a medium containing biotin, only traces of other fatty acids are found in the cell lipids, showing almost complete inhibition of fatty acid synthesis. Both oleic and **cis-vaccenic acids** have the same effect except for the presence of **C$_{19}$** CFAs derived from them.

Kodiecek (1963) pointed out that branched chain, polyunsaturated and CFAs might all be expected to resist being packed very closely in a surface film and thus give elasticity preventing membranes from being rigid and brittle. By
comparison of the membranes from *L. casei* and striated muscle, he found a remarkable similarity in fatty acid composition, except that, instead of lactobacillic acid, linolenic acid was present in muscle membrane. Phospholipids containing either cyclopropane or unsaturated fatty acids are similar in their solubility characteristics in polar solvents and in their tendencies to form micellar dispersions (Law et al., 1963; Rothfield and Pearlman, 1966). Also, X-ray studies show that CFAs and their corresponding monoenoic acids have similar shapes and crystal structures (Craven and Jeffrey, 1960). In membranes practically devoid of unsaturated fatty acid residues, the CFAs abundant in several species of bacteria may take over the function of regulating the distance between the paraffinic chains or the liquid crystalline state of lipids in the membrane (Van Deenen, 1965). The same author reported that several enzymes require phospholipids for their normal function and the CFA-containing phosphatidylethanolamine prepared from *E. coli* revealed a pressure-area curve which was hardly distinguishable from the characteristic curve given by a film of (stearoyl-oleoyl)-L-α-phosphatidylethanolamine. In plants, lipid esterified with long chain CFAs could contribute to the physiological adaptation of early spring and drouth-tolerant plants (Kuiper and Stuiver, 1972). It is known that chilling resistance in plants is correlated with high flexibility of mitochondria and with a high degree of unsaturation of the mitochondrial lipids (Lynos and Raison, 1970; Lynos et al.,
1964). It is not known, however, why CFAs should be preferred to monoenoic acids or what advantages occur to the organism in performing the energetically expensive reaction involved in their synthesis.

No completely satisfactory proposal concerning the function of CFAs can be made with information presently available. In bacteria, their synthesis may serve to remove some undesirable metabolic product such as S-adenosylmethionine or unsaturated fatty acids (Law et al., 1963), but this would seem less economical than the well-controlled bacterial metabolism usually permits. Weinbaum and Panose (1966) found that filamentous forms of *E. coli* B contained less CFAs than did the normal cell and suggested that these acids might play an important structural role in the cell envelope. Prolonged culture of certain *Vibrio cholerae* strains gives rise to variants termed "rugose", which exhibit unusual resistance to adverse environmental conditions. Brian and Gardner (1968) noticed that when both parent and rugose cultures were recultured, fatty acids of rugose variants were similar but differed from those of the parent strains. All contained large quantities of *C*₁₇ and *C*₁₉ CFAs which were undetected in parent cultures. Thence, they postulated that the presence of CFAs in rugose *V. cholerae* is related to the survival characteristics of these variants. It has been also suggested that they may be semi-stable storage forms of 1-carbon fragments or that they may function somewhat as sterols do in higher forms in
contributing to membrane elasticity, but both of these concepts are clouded by the knowledge that CFAs accumulate mostly in the late exponential and early stationary phases of growth (O'Leary, 1967). The same author argued that the suggestion that they are merely a dead-end aging phenomenon or the result of some sort of detoxifying activity is inconsistent with these acids apparent indispensibility in those organisms that contain them, since there are organisms that fail to grow if prevented from either synthesizing or absorbing them. Alternatively, Law et al. (1963) have suggested that unsaturated fatty acids in the membranes of bacteria are labile to either oxygen in aerobic bacteria or free radicals in anaerobic organisms, and since bacteria are not known to contain lipid antioxidants, saturation by formation of a cyclopropane ring could prevent the lipid peroxidation. There is obviously something about the CFAs' physiological role that has not yet been seen and much work is still needed to solve the mystery of their function.

**Tetrahymena pyriformis**

**Characteristics**

*Tetrahymena pyriformis* is a free living, widely distributed ciliate protozoan, the habitat of which is fresh water, ranging from springs, ditches, creeks, rivers, ponds and lakes to thermal springs, and perhaps even salt marshes and soil (Hill, 1972). It has an average of cell size of 50 x 30 μ. However,
the cell size varies according to the growth conditions. Cells transferred during the logarithmic phase to a non-nutrient medium can divide in absence of cell growth. This results in smaller size cells (Cameron and Terebey, 1967). The size also decreases as the temperature of the growth medium increases up to 29°C (optimum temperature), above which the size starts to increase (Thormar, 1962). When grown in a range of 0.5%-8% of proteose peptone, the cell size becomes a function of concentration up to 4%, beyond which it decreases (Huddleston et al., 1964). This change of size with nutrient concentration is independent of the pH. The influence of pH on multiplication rate is small over a broad range of pH, 5.65-8.40 (Prescott, 1958). Slightly below and above this range the rate is severely reduced.

An ultrastructural study of ingestion and digestion has been made by Elliott and Clemmons (1966). Food vacuoles form in the buccal cavity and move into the cytoplasm. Pinocytotic vacuoles may originate from differentiated regions of the pellicle and also move into cytoplasm. Neither food vacuoles nor pinocytotic vacuoles contain hydrolytic enzymes. Once the pinocytotic vacuoles receive hydrolases from the primary lysosomes, they fuse with the food vacuoles and degradation of the contained food materials is initiated. The same author found that in stationary growth phase cells, autophagic vacuoles appear containing mitochondria and cellular debris. Primary lysosomes may coalesce with them and ultimately digest
the contents. Some of the soluble nutrients may enter directly through the cell wall (Seaman, 1961).

In a defined medium, this organism requires at least 10 amino acids, 6 vitamins, guanine and uracil, in addition to some inorganic salts (Hill, 1972). Media separated from T. pyriformis cultures were found to contain secreted acid phosphatase, ribonuclease, deoxyribonuclease, proteinases and amylase (Muller, 1967). Those enzymes differ from the intracellular enzymes in their thermal sensitivity, pH optima and site of action.

Lipid composition

T. pyriformis grown in defined media shows no requirement for fatty acids or for any other lipid compound (Elliott, 1950). Carter and Gaver (1967) reported that lipid comprises 8% of the dry weight of T. pyriformis. On the other hand, Taketomi (1961) reported it as 17.4% of the dry weight, while Kozak and Huddleston (1966) as more than double this amount. The amount of lipid per cell changes with the age of the population. The total cellular lipid increases throughout the logarithmic and early stationary phases, but decreases sharply after 72 hours (Everhart and Ronkin, 1966). This change is reflected morphologically in that the percentage of cells containing neutral lipid granules increases in the early stationary phase, decreases in the middle of the stationary phase, and increases again in the late stationary phase and
decreases later (Allison and Ronkin, 1967; Thompson, 1967). The extractable lipids of logarithmic phase cells consist primarily of phospholipids (68%) and neutral lipids (28%), while free fatty acids are present in negligible amounts (Erwin and Bloch, 1963). Phosphatidylethanolamine constitutes 54% of the phospholipids and phosphatidylcholine constitutes 30% while only a small amount of phosphatidylserine is detected. Phosphatidylcholine of the classical type is replaced to a significant degree by the glycerol ether analogue, and the phosphatidylethanolamine also contains a considerable amount of bound glycerol ethers as well as 2-aminoethylphosphonic acid (Thompson, 1967). As the culture approaches stationary phase, the accumulation of neutral lipids may approach on a weight bases the phospholipid content (Thompson, 1967). Triglycerides constitute about 90% of the neutral lipids (Erwin and Bloch, 1963). *T. pyriformis* also contains a small amount of waxes, plasmalogen (Taketomi, 1961), sphingolipids (Taketomi, 1961; Carter and Gaver, 1967) and fatty acid esters of methanol and ethanol (Ming Chu et al., 1972). An unusual lipid, 1,14-docosyl disulfate, constitutes a major component (5-10%) of the total fatty acids in *T. pyriformis* (Haines, 1965).

A typical fatty acid pattern of *T. pyriformis* is given in Table 1. In addition to those in the table, Erwin and Bloch (1963) found an unidentified fatty acids that constitute 3.6% of total fatty acids. Also, Muller et al. (1959) reported
Table 1. Relative fatty acid composition (% of total) of *T. pyriformis*

<table>
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<td>1.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Oleic 18:1(9)</td>
<td>8.7</td>
<td>26.9</td>
</tr>
<tr>
<td>Linoleic 18:2(9,12)</td>
<td>17.9</td>
<td>19.7</td>
</tr>
<tr>
<td>γ-Linolenic 18:3(6,9,12)</td>
<td>37.7</td>
<td>13.3</td>
</tr>
<tr>
<td>Arachidic 20:0</td>
<td>&lt; 1.0</td>
<td></td>
</tr>
<tr>
<td>Eicosenoic 20:0</td>
<td>1.0</td>
<td>1.7</td>
</tr>
</tbody>
</table>
small amounts of saturated \( C_{22} (0.7\%) \), \( C_{24} (0.3\%) \) and \( C_{26} (0.1\%) \) and also unsaturated \( C_{22} (4.8\%) \) and \( C_{24} (1.2\%) \) fatty acids in addition to those listed in Table 1. This fatty acid pattern can be altered by supplementation of the growth medium with different nutrients (Shorb, 1963). The fatty acids of those grown in normal media and that supplemented with acetate are largely the straight-chained even-numbered acids. When the medium is supplemented with sodium propionate, small amounts of odd-numbered fatty acids appear, namely \( C_{13}, C_{15}, C_{17} \) and \( C_{17:1} \). With the addition of isobutyric acid, branched-chain higher acids appear. These are iso-\( C_{14}, \) iso-\( C_{16} \) and iso-\( C_{18} \); the iso-\( C_{16} \) being in highest concentration. Supplementation with alpha-methyl-n-butyric acid resulted in the synthesis of the branched anteiso-\( C_{13}, \) anteiso-\( C_{15}, \) anteiso-\( C_{17} \) and anteiso-\( C_{19} \) fatty acids. The ratio of fatty acids also alters with culture age (Erwin and Bloch, 1963). With increasing age of the culture, the ratio of monounsaturated fatty acids to their saturated analogues decreased while the relative amount dienoic and trienoic acids remained constant. Five environmental factors—iron availability, oxygen tension, sterol supplementation, temperature and the overall medium composition—have been demonstrated to alter the fatty acid content of the phospholipids in \textit{T. pyriformis} (Conner and Koroly, 1972). Iron supplementation or lowering the temperature increases the proportional amount of \( C_{18:2}(6,11) \), while iron and a higher degree of aeration increases both \( C_{18:2}(6,11) \) and
Ergosterol also increases the $C_{18:2}(6,9)$, but it causes a decrease in the specific class of sphingolipids which contains saturated fatty acids. When T. priformis is grown in a defined medium, it lacks alpha-hydroxy fatty acids in the sphingolipids, but contains these acids when grown on proteose peptone. Another study of the influence of environmental factors on lipid composition of T. pyriformis (Knuese and Shorb, 1966) showed that an unknown polyunsaturated compound is present in the cells grown in the light but not in the dark.

**Metabolism of fatty acids**

Beta-hydroxybutyrate dehydrogenase, an enzyme that serves in the oxidation of fatty acids, is present in T. pyriformis (Koehler and Fennell, 1964; Helmer, 1968) in mitochondria. It is NAD-linked (Conger and Eichel, 1965). NADP cannot replace NAD for the conversion of beta-hydroxybutyrate to acetoacetate catalyzed by this enzyme. Differential inhibition of the beta-oxidation pathway by malonate and of alpha-oxidation by imidazole in *in vivo* experiments (Avins, 1968) suggests that both pathways are present in this organism. T. pyriformis grown in the presence of the unnatural fatty acids 11,14-eicosadienoate, 8,11,14-eicosatrienoate, 11-eicosenoate or 11-octadecenoate incorporates them into the natural lipids and phospholipids (Lees and Korn, 1966). Some of these fatty acids can be further desaturated or elongated.
Phylogenetic relationship

In an attempt to relate *T. pyriformis* to one of the known kingdoms, Hill (1972) summarized all the information known about this organism as follows:

There are strong arguments in favour of calling them animals. When viewed under the microscope, they are seen to be motile, to ingest food, and to have no chloroplasts. They have the same amino acid requirements as man and rat, and the vitamin requirements for all three are quite similar. Tetrahymena contains, as do higher animals, glycogen as a storage form of carbohydrate; it breaks down the glycogen anaerobically to lactate. The glycogen is formed from uridine diphosphate-glucose, a precursor of glycogen in animals but not in plants. The biosynthesis of phosphatidylserine proceeds in a manner similar to that in animals, but not to that in bacteria; and the ribonucleic acid polymerase is similar to that in animals but not to that in bacteria. The organism also possesses hemoglobin and probably contains N-phosphoryl arginine as a phosphagen. Neither substance is found in plants.

This formidable evidence is not conclusive, however. Tetrahymena require lipoate; animals are able to synthesize this vitamin. The ciliate makes a vitamin B₁₂-like compound, but animals require vitamin B₁₂. The arginine dihydrolase enzyme, used by *T. pyriformis* to convert arginine to citrulline, has been found in bacteria and yeast, but not in animals. The ciliate also has a bacterial-type cytochrome c. Finally, *T. pyriformis*, like plants, has an operative glyoxylate cycle and contains a pentacyclic triterpenoid, both of which have been found only in plants.

He concluded that on the basis of this evidence, one cannot describe *Tetrahymena* as either plant or animal.
EXPERIMENTAL

Equipment

Gas liquid chromatographic analyses were performed with a Varian Aerograph model 1520 gas chromatograph equipped with a flame ionization detector and a 1/8 in. x 6 foot stainless steel column packed with 3% OV-1 on chromosorb Q 100/120 mesh (Applied Science Laboratories, State College, Pennsylvania). The temperature of the column was 200 C, and the nitrogen and hydrogen flow rates were 25 ml/min. The preparative gas chromatograph used for purification of the chemically synthesized CFAs was an Aerograph model A-100 equipped with a thermal conductivity detector and a 1/4 in. x 7 foot stainless steel column packed with 20% DEGS on chromosorb W 60/80 mesh (Applied Science Laboratories). The temperature of the column was 180 C and the helium flow rate 40 ml/min.

For isotope counting, a Packard Tri-Carb Scintillation spectrometer model 3310 equipped with automatic control model 577 and a refrigeration unit was used. An Atlas CH4 mass spectrometer, operated by chemistry department at Iowa State University, was used for further identification of the chemically synthesized CFAs.

Materials

Zinc-copper couple, methylene iodide, and nicotinamide were products of Eastman Kodak Co., Rochester, N.Y. Standard
fatty acid methyl esters having even carbon numbers of 8-20, the methyl esters of cis-9,10-methyleneoctadecanoic, vaccenic, and oleic acids and methyl oleate-U-\(^{14}\)C (750 m Ci/m mole) were purchased from Applied Science Laboratories. Acetone-1,3-\(^{14}\)C (26.8 m Ci/m mole) was obtained from Amersham/Searle, Arlington Heights, Ill. and palmitic acid-U-\(^{14}\)C (0.05 m Ci/0.016 mg) from New England Nuclear Corp., Boston, Mass. 1,4-Bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP), 2,5-diphenyloxazole (PPO) and 2,5-bis-2-(5-tert-butylbenzoxazolyl)-thiophene (BBOT) were purchased from Packard Instrument Co., Inc., Downers Grove, Ill. and Cab-O-Sil from Research Products International Corp., Elk Grove Village, Ill. Adenosine triphosphate (ATP), \(\beta\)-nicotinamide adenine diphosphonucleotide (NAD), avidin, and dithiothreitol were obtained from Sigma Chemical Co., St. Louis, Mo. Coenzyme A (CoA) and DL-carnitine hydrochloride were obtained from Calbiochem, Los Angeles, Cal. Bovine serum albumin fraction V (BSA) was purchased from Pentex Inc., Kankakee, Ill. All solvents were reagent grade and were redistilled.

Methods

CFA synthesis

Synthesis of cis-9,10-methyleneoctadecanoic acid

Iodoform was prepared from acetone as described by Shriner and Fuson, (1948) with slight modification. To 0.2 ml acetone in a test tube,
3 ml of 10% NaOH was added and agitated. This was followed by gradual addition and shaking of 10.5 ml iodine-potassium iodide solution (prepared by adding 20 g potassium iodide and 10 g iodine to 80 ml water). The excess iodine was removed by adding a few drops of 10% NaOH solution with shaking. The iodoform crystals formed were washed several times with distilled water and transferred to a 50 ml pear-shaped flask.

Methylene iodide was prepared from iodoform by modification of the method described by Adams and Marvel (1941). A 13 mm magnetic stirring bar was dropped into the flask containing the iodoform crystals. Two ml of sodium arsenite solution (prepared from 27.4 g arsenious oxide, 53.2 g sodium hydroxide, and 260 ml water) was added and a small reflux condenser was fitted to the flask. The flask was immersed in a water bath placed on a magnetic stirrer-hot plate and stirring was started. The water bath temperature was increased gradually to 65-70 C, then kept constant in that range. Ten ml of the sodium arsenite solution was then added from the top of the condenser in 2 ml fractions during the course of 30 minutes. The reaction was allowed to go for one more hour at 65-70 C. When the reaction was completed, the flask was allowed to cool for a short time, then several ml of anhydrous diethyl ether was added from the top of the condenser to wash it down. The condenser was removed and the contents of the flask were stirred. The ether layer was then filtered through Whatman phase separating filter paper. The aqueous layer was re-
extracted two times with diethyl ether and filtered through the same filter paper. The combined ether extracts were dried overnight over anhydrous magnesium sulfate, then filtered and the filtrate which contained the methylene iodide was brought to about 0.5 ml under vacuum in a rotary evaporator.

**cis-9,10-methyleneoctadecanoic acid** was prepared from methylene iodide and methyl oleate as described by Simmons and Smith (1959), but on a small scale and with modification of their purification method. The diethyl ether containing methylene iodide was transferred to a 5 ml round bottom flask containing 200 mg methyl oleate. The total volume of the mixture was about 1.0 ml. Six-tenths of a g zinc-copper couple and 0.1 g iodine were added, followed by dropping a 13 mm magnetic stirring bar into the flask. The contents were stirred for 48 hours under reflux. After it cooled down, the reaction mixture was filtered through a sintered glass filter with suction and the reaction flask washed several times with diethyl ether and the washings filtered. The filtrate was successively washed with 5% HCl, water, and 5% sodium sulfite solution. It was then brought to a small volume under vacuum in a rotary evaporator and dried completely under nitrogen. The fatty acid methyl esters obtained were redissolved in a small amount of hexane.

Argentation thin layer chromatography plates were prepared by allowing a saturated solution of silver nitrate in 95% methanol to ascend 20 x 20 cm plates covered with silica
gel G, 0.5 mm (Mahadevan, 1967). The plates were then removed, dried for 30 minutes at room temperature and activated at 110°C for one hour. They were left to cool and the hexane solution of the fatty acid methyl esters was streaked on several such plates. The plates were developed in ether-hexane (7:93 V/V). cis-9,10-methyleneoctadecanoic acid methyl ester was also spotted separately on each plate as a reference. After the plates became dry, they were sprayed with aqueous Rhodamine-6-G solution (120 mg/l), dried, and the fatty acid methyl esters were visualized under UV light. The spots corresponding to CFA were scraped from the plates and eluted with diethyl ether. The eluent was brought to dryness and the residue was redissolved in hexane. The identity of the product was verified by analytical GLC. All the steps in argentation thin layer chromatography were performed in the dark.

Preparative GLC was used to isolate the two fatty acid methyl esters detected by the analytical gas liquid chromatography. They were recovered by attaching to the exit channel of the gas chromatograph a calcium chloride tube loosely packed with pyrex wool that has been wetted with petroleum ether (James, 1960). Each fatty acid methyl ester was extracted from the pyrex wool by flushing the tubes with petroleum ether. After that the solvent was removed and the sample subjected to analysis by mass spectroscopy.
Synthesis of $[{\text{11,12-methylene}}^{14}\text{C}]$ cis-11,12-methylene-octadecanoic acid Two batches of this fatty acid were prepared. The first batch was prepared as described for the preparation of cis-9,10-methyleneoctadecanoic acid with the following modification.

The sealed tube containing acetone-1,3-$^{14}$C (250 m Ci) was cooled in liquid nitrogen and opened while it was in liquid nitrogen. Nonlabelled acetone (0.2 ml) was added and the tube was taken out of liquid nitrogen. Also, methyl vaccenate was used instead of methyl oleate.

After purification by preparative GLC, $[{\text{11,12-methylene}}^{14}\text{C}]$ cis-11,12-methyleneoctadecanoic acid methyl ester was dissolved in hexane and its specific activity was calculated. This was done by determining the amount of CFA methyl ester in a known aliquot using the analytical GLC technique (Kruppa and Doyle, 1972) and then counting the radioactivity in another aliquot. CFA methyl ester was then saponified by the addition of 6.0 ml of 5% KOH in 50% methanol and refluxing for 2 hours. This was followed by the addition of 1.5 ml of 6.0 N HCl and extraction three times with diethyl ether. The solvent was removed and the residue was redissolved in 95% ethanol.

The second batch of $[{\text{11,12-methylene}}^{14}\text{C}]$ cis-11,12-methyleneoctadecanoic acid was prepared and assayed for radioactivity as the first batch with two differences. The radioactive acetone was diluted with only 0.1 ml of unlabelled
acetone and the amount of methyl vaccenate used was 100 mg.

Synthesis of cis-9,10-methyleneoctadecanoic acid from oleic acid-U-\textsuperscript{14}C methyl ester and methylene iodide

This was prepared by the Simmons-Smith reaction as described before. The reaction reagents used were: zinc-copper couple 0.22 g, iodine 0.05 g, methyl iodide 0.53 g, methyl oleate 0.01 g, and oleic acid-U-\textsuperscript{14}C methyl ester 0.01 g. All the steps for purification, measuring the specific activity and saponification, were as described for the synthesis of the CFA labelled at the ring.

Organisms used and their growth conditions

The stock culture of \textit{E. coli} (BFG) was obtained from Dr. J. Horowitz, Iowa State University. Cells were grown in nutrient agar slants for 24 hours at 37 C before they were transferred to the synthetic medium specified by Davis and Mingioli (1950). The composition of the synthetic growth medium is as follows:

\begin{align*}
\text{g/100 ml H}_2\text{O} \\
\text{K}_2\text{HPO}_4 & \quad 0.7 \\
\text{KH}_2\text{PO}_4 & \quad 0.3 \\
\text{Sodium citrate \cdot 3H}_2\text{O} & \quad 0.05 \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & \quad 0.01 \\
(\text{NH}_4)_2\text{SO}_4 & \quad 0.1 \\
\text{Glucose} & \quad 0.2
\end{align*}
Glucose and magnesium sulfate were sterilized separately and added to the sterile growth medium in a 250 ml Erlenmeyer flask prior to inoculation with \textit{E. coli}. After inoculation, the flask was incubated at 37 °C for 24 hours in a gyratory shaker.

\textit{Ochromonas danica} stock culture was obtained from American Type Culture Collection, Rockville, Maryland. The cells were maintained by growing them in culture tubes containing fluid thioglycollate medium (Difco Laboratories, Detroit, Michigan) at 18 °C. Whenever needed, 250 ml flasks containing 100 ml of a chemically defined medium (General Biochemicals, Inc., Chagrin Falls, Ohio) recommended by Aaronson and Baker (1959) were inoculated with 2 ml of the stock culture. After growing for 2 days at room temperature, 2 ml was transferred to fresh chemically defined medium. The medium for growing \textit{O. danica} on \textit{E. coli} cells was supplemented with the following minerals that are required for a healthy growth of \textit{O. danica} (Aaronson and Baker, 1959):

\[
\begin{align*}
\text{mg/100 ml H}_2\text{O} \\
\text{Nitrilotriacetic acid} & \quad 20.0 \\
\text{KH}_2\text{PO}_4 & \quad 30.0 \\
\text{MgCO}_3 \text{ (basic)} & \quad 40.0 \\
\text{CaCO}_3 & \quad 5.0 \\
\text{NH}_4\text{Cl} & \quad 50.0 \\
\text{MgSO}_4 & \quad 100.0
\end{align*}
\]
T. pyriformis (GL) stock culture was obtained from Dr. D. Outka, Iowa State University. Cells were maintained by growing in culture tubes containing 2% proteose peptone and 0.1% yeast extract at 18°C. Whenever needed, cells were recultured twice in fresh medium and each grown for 24 hours at room temperature.

When T. pyriformis cells were grown on an E. coli-containing culture, the culture used to grow E. coli was supplemented with the following minerals required by T. pyriformis.

\[
\begin{array}{ll}
\text{mg/100 ml } H_2O \\
\text{CaCl}_2 \cdot 2H_2O & 5.0 \\
\text{Fe(NH}_4\text{)}_2\text{SO}_4 \cdot 6H_2O & 2.5 \\
\text{FeCl}_3 \cdot 6H_2O & 0.125 \\
\text{MnCl}_2 \cdot 4H_2O & 0.05 \\
\text{ZnCl}_2 & 0.005 \\
\text{CuCl}_2 \cdot 2H_2O & 0.5 \\
\end{array}
\]
Biodegradation of CFAs in vivo

By growing O. danica on E. coli as a source of CFAs

Four 250 ml Erlenmeyer flasks containing 100 ml each of mineral medium required by O. danica were sterilized and inoculated with 10 ml of E. coli-containing fluid culture medium and 5 ml of O. danica-containing culture medium. A 40 ml sample was taken from the first flask immediately after inoculation and considered as zero growth time. The flasks were left at room temperature and the same amounts were taken from the second flask after 3 days and from the third flask after 5 days. After the addition of 10 drops of 6.0 N HCl, the samples were centrifuged in a clinical centrifuge at top speed for 30 minutes and the supernatant discarded. The sediment was re-suspended in 2 ml of water.

The method described by Dryer (1970) for the extraction of lipids from blood was found very convenient and was used as a standard procedure for the extraction of lipids from all cultures. The 2 ml sample was shaken with 10 ml of methanol, left for 5 minutes, another 10 ml of methanol added and shaken again. After leaving for another 5 minutes, 20 ml of chloroform was added to the sample, it was shaken well and then centrifuged for 15 minutes in a clinical centrifuge. The extract was then washed with 0.2 its volume of 0.1 M NaCl solution and the solvent removed.

The lipid residue left was saponified with 6.0 ml of 10% KOH in 50% methanol under reflux for 2 hours. After it
cooled down, it was acidified with 3.0 ml of 6.0 N HCl and the fatty acids were extracted three times with diethyl ether (10 ml at a time) and the extracts pooled. This saponification and extraction procedure was used whenever fatty acids were isolated from lipid residues obtained from cells or culture medium. The solvent was removed and the residue redissolved in diethyl ether and transferred to a methylation flask where the solvent was removed again.

Fatty acids were methylated with 2 ml of 2% HgSO$_4$ in absolute methanol at 55°C for 2 hours (Selvey, 1960). One ml of water was added and the flask was agitated. Hexane (0.25 ml) was added, followed by the addition of enough water to bring the organic solvent to the neck of the flask, with shaking after each addition. Three ul of the hexane solution was injected into the analytical gas chromatograph.

The fourth flask was left for 5 days at room temperature. A 40 ml sample was taken carefully from the top of the culture medium and centrifuged at a medium speed in a clinical centrifuge for 5 minutes. The sediment was washed twice with distilled water and resuspended in 2 ml of water. One drop was taken for microscopic examination and lipid was extracted from the sample and treated as discussed before for the isolation, methylation and identification of fatty acids.

By growing *T. pyriformis* on *E. coli* as a source of CPAs *E. coli* cells were grown in 100 ml medium containing the minerals required by *T. pyriformis* at 37°C for 24 hours. One ml of
a T. pyriformis culture was added and a 4 ml sample was taken and considered as zero growth time. Protozoan cells were allowed to grow at room temperature and additional 4 ml samples were taken after 3 and 5 days with shaking of the flask before taking each sample.

Lipids were extracted from each sample as before except that double the amount of solvent was used. The methyl esters of the fatty acids were prepared, extracted with 0.25 ml hexane and 3 ul injected into the analytical gas chromatograph.

The same experiment was repeated, except that pent-4-enoic acid was added at the time of the addition of the T. pyriformis culture to the E. coli culture in two separate flasks. The final concentration of pent-4-enoic acid was 0.01 and 0.1 mM. Another flask was used as a control in which no pent-4-enoic acid was added. Samples of 2.0 ml were taken at zero time and after 4 days of incubation and treated as others.

By growing T. pyriformis on a growth culture supplemented with [11,12-methylene-14C] cis-11,12-methyleneoctadecanoic acid T. pyriformis cells were grown as shown in Figure 1. A 250 ml Erlenmeyer flask with a side arm covered by a serum stopper (no. 4) and attached to two U-shaped tubes (no. 3 and 5) packed with cotton was used for their growth. The flask contained 100 ml of 1% proteose peptone and 0.1% yeast extract and 240,000 dpm of [11,12-methylene-14C] cis-11,12-methylene-octadecanoic acid (0.028 m Ci/m mole). The CFA was added
Figure 1. The system used for growing *T. pyriformis* in culture medium containing [\(11,12\)-methylene-\(^{14}\)C] \textit{cis}-11,12-methyleneoctadecanoic acid; (1) air inlet; (2) contained 300 ml saturated Ba(OH)\(_2\) solution; (3 and 5) packed with cotton; (4) contained culture medium supplemented with the CFA; (6) contained 5 ml of 25% KOH; (7) contained ascarite.
dissolved in 20 ul of 95% ethanol. All the three containers were sterilized while they were attached together. The culture medium was inoculated with 1.0 ml of \textit{T. pyriformis} culture through the serum stopper using a hypodermic needle and syringe. Flask no. 2 contained 300 ml saturated Ba(OH)$_2$ solution to trap CO$_2$ from the air while flask no. 5 contained 5 ml of 25% KOH to trap $^{14}$CO$_2$ released from the culture. A slow current of air was allowed to go through and the culture was left at room temperature. Samples of 0.5 ml were taken from flask no. 6 immediately after inoculation, after 2 days, and then every day for another 5 days. Samples were assayed for radioactivity in 15 ml Cab-O-Sil scintillation gel (Heintz et al., 1968). The counts were corrected for the total volume of solution in flask no. 6.

At the seventh day of growth, a 40 ml sample was taken from flask no. 4 and divided into two equal parts which were chilled in ice. Protozoa cells were collected by centrifugation for 5 minutes at 1000 x $g$ and 0-2 C. The cells were resuspended in 1.0 ml water and lipids were extracted twice as described before. Each extract was assayed for radioactivity after each extraction.

Glycogen was isolated from the sediment as described by Rendina (1971), but on a smaller scale. This was done by adding 5 ml of 50% KOH to the sediment in each tube and heating the mixture in a boiling water bath for 45 minutes. Five ml of water was added to each tube followed by 30 ml of 95%
ethanol. The contents of the tubes were mixed, allowed to stand for 30 minutes and then centrifuged. All centrifugations were carried out in a clinical centrifuge unless otherwise specified. The sediment in both tubes was pooled in 7.5 ml of 10% trichloroacetic acid. After centrifugation, the sediment was discarded and 30 ml of 95% ethanol was added to the supernatant and left over night for glycogen to flocculate. Glycogen was collected by centrifugation, dried and redissolved in 1.0 ml of 1.0 N KOH. A 0.5 ml portion was used to assay for radioactivity in 10 ml Cab-O-Sil scintillation gel.

By incubating T. pyriformis with [11,12-methylene-14C] cis-11,12-methyleneoctadecanoic acid Three flasks (2 l each) containing 1.0 l of 2% proteose peptone and 0.1% yeast extract each were inoculated with 2.0 ml of T. pyriformis culture. The cells were allowed to grow at 25 C for 2 days and were collected by centrifugation at 1000 x g for 10 minutes. After washing them twice with a medium that contained 0.15 M NaCl and 0.02 M KH2PO4 at pH 7.0, the cells were resuspended in 25 ml of the same medium. Three ml-fractions of this suspension were incubated with [11,12-methylene-14C] cis-11,12-methyleneoctadecanoic acid (114090 dpm, 0.077 m Ci/m mole). The incubation was done in Warburg flasks that were stoppered with serum stoppers and with 2 filter papers (0.5 in. in diameter) in the center well. Each flask contained 36x10^5 cells and the labelled CFA was introduced dissolved in 10 ul
of 95% ethanol. The incubation was carried out at 25 °C in a metabolic shaker with or without 10 mM malonate for 2, 4 and 6 hours.

At the end of each period of time mentioned, one flask with and one without 10 mM malonate was removed from the water bath. Three-tenths of a ml of 6.0 N H₂SO₄ was injected through the serum stopper into the main incubation medium and 0.1 ml of 25% KOH onto the filter papers. The flasks were shaken for another hour to collect the ¹⁴CO₂ released from the medium. The filter papers were removed from each flask and dropped into liquid scintillation vials and left to dry over night. They were opened flat and 10 ml Bray's liquid scintillation solution (Bray, 1960) was added and radioactivity was measured.

To the main incubation medium, 1.0 ml of 2.5 N KOH was added and the cells were broken down by sonication and left over night at 37 °C. The samples were deproteinized as described by Malawer and Powell (1967). The following reagents were added with agitation of the tubes after each one: 4 ml of 5% BaCl₂, 4 ml of 0.3 N Ba(OH)₂ and 4 ml of 5% ZnSO₄. Also, 20 u mole of sodium acetate and of sodium propionate were added to each tube. The tubes were centrifuged in a clinical centrifuge and the supernatant was acidified with 0.8 ml of 6.0 N H₂SO₄, then distilled in a small distillation apparatus to half its volume. The distillate was collected in a tube containing 1.0 ml of 2.5 N KOH. Water was added to the distillation flask to restore the sample to its original volume.
and it was distilled to half its volume again. The combined distillate was brought to about 0.5 ml under vacuum in a rotary evaporator. It was then acidified with 0.3 ml of 18 N H₂SO₄, transferred onto a silicic acid column as described by Ramsey (1963), and the column was eluted with 60 ml benzene, 100 ml chloroform and 100 ml chloroform containing 1% tert-butanol. All the solvents were equilibrated with 25 ml of 0.5 N H₂SO₄ per l. The column was eluted under 2 psi of nitrogen and 10 ml fractions were collected.

Fractions containing acetic and propionic acids were located by titration with 0.01 N ethanolic KOH. Two drops of 0.1% thymolphthalein in ethanol was added as indicator and nitrogen was bubbled through the samples during titration. After titration, 2 ml of 0.2 N ethanolic KOH was added to each vial and the contents were dried under nitrogen. Five drops of 2.0 N acetic acid was added to each vial to dissolve the salts and the radioactivity was measured in 10 ml of Bray's liquid scintillation solution.

**Fatty acid oxidation in vitro**

**Culture and preparation of T. pyriformis cells** Two ml of T. pyriformis culture was inoculated into 1 l of medium containing 2% proteose peptone and 0.1% yeast extract in a 2 l Erlenmeyer flask. Six such flasks were prepared for each experiment. The flasks were incubated for 48 hours at 25 C. Then the cells were collected by centrifugation for 10 minutes
at 1000 x g, and washed twice with a medium for the preparation of a particulate fraction of the cell homogenate (pellet), described by Kobayashi (1965). The medium contained 0.35 M mannitol, 0.05% BSA, 0.1 m M EDTA and 1.0 m M Tris-HCl buffer at a final pH of 7.2. All centrifugations were performed at 0-2 C.

Preparation of pellet This was prepared as described by Kobayashi (1965) for the preparation of mitochondria from T. pyriformis with some modification. All steps for the preparation of the pellet were performed at 0-2 C. The cells were suspended in 20 ml of the preparation medium. Ten ml portions were gently homogenized in a glass homogenizer with a tightly fitting teflon pestle. The homogenate was immediately centrifuged at 10,000 x g, for 5 minutes and the supernatant was discarded. The resulting sediment was resuspended in 40 ml of the preparation medium and centrifuged at 200 x g for 2 minutes to remove the unbroken cells. The supernatant was then centrifuged at 10,000 g for 5 minutes and the supernatant was discarded. The sediments were then pooled in one tube and washed once with 30 ml of the preparation medium. The resulting pellet was resuspended in a small amount of the same medium such that 0.5 ml contains approximately 20 mg pellet protein. The appropriate amount of this suspension was added to the incubation medium for fatty acid oxidation.
**Incubation medium for fatty acid oxidation**

Unless otherwise specified, the reaction was carried out in a standard mixture containing the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>250</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.2</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>5.0</td>
</tr>
<tr>
<td>KCl</td>
<td>10.0</td>
</tr>
<tr>
<td>ATP</td>
<td>1.0</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>2.5</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>25.0</td>
</tr>
<tr>
<td>CoA</td>
<td>0.1</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>1.0</td>
</tr>
<tr>
<td>BSA</td>
<td>0.014</td>
</tr>
<tr>
<td>Phosphate buffer (pH 7.4)</td>
<td>20.0</td>
</tr>
</tbody>
</table>

All incubation medium reagents were prepared in a higher concentration and mixed in the appropriate amounts. A stock solution that contained EDTA, MgCl₂ and KCl at a final pH of 7.3 was used. NAD⁺ was prepared in nicotinamide solution and the pH was adjusted to 7.0. The pH of the ATP solution was also adjusted to 7.0. CoA was prepared in dithiothreitol solution.

**Incubation of labelled fatty acids with pellet**

Incubations were performed in Warburg flasks closed with serum stoppers. When $^{14}\text{CO}_2$ was to be collected, 2 filter papers (0.5 in. in diameter) were placed in each center well. The labelled fatty acid was added dissolved in 10 μl of 95%
ethanol. The amount of labelled substrates used for different experiments were: palmitic acid-U-$^{14}$C 240,000 dpm (0.05 m Ci/0.016 mg), [11,12-methylene-$^{14}$C] cis-11,12-methyleneoctadecanoic 114,090 dpm (0.077 m Ci/mole), cis-9,10-methylene-octadecanoic acid with all the carbons labelled with $^{14}$C except the methylene carbon 168,070 dpm (0.15 m Ci/m mole).

The amount of pellet protein used was determined by the biuret method (Gornall et al., 1949) and flasks were incubated for 4 hours at 25°C in a metabolic shaker. At the end of the incubation time 0.1 ml of 25% KOH was injected onto the filter papers and the reaction was stopped by injection of 0.3 ml of 6.0 N H$_2$SO$_4$ into the medium. The flasks were shaken for one more hour to collect $^{14}$CO$_2$.

In some of the incubation media, malonate, carnitine and/or avidin were also added. The pH of a concentrated solution of each one was adjusted to 7.0 before addition to the incubation medium.

Controls were prepared by the addition of 0.3 ml of 6.0 N H$_2$SO$_4$ immediately after the addition of the substrates.

Isolation and analysis of metabolic products The filter papers were transferred to liquid scintillation vials, left to dry over night, opened flat and radioactivity corresponding to $^{14}$CO$_2$ was counted in 10 ml of Bray's liquid scintillation solution.

One ml of 2.5 N KOH was added to the incubation medium and it was left over night at 37°C. Acetate and propionate
were then isolated and assayed for radioactivity as described earlier.

In some samples where $^{14}$CO$_2$ was not collected, the incubation was stopped by the addition of 0.3 ml of 6.0 N H$_2$SO$_4$ to the medium, followed by 1.0 ml of 2.5% KOH. The samples were then treated in the same way as the others.

**Position of the labelled carbon in acetate-$^{14}$C** Four experiments were performed in which [11,12-methylene-$^{14}$C] cis-11,12-methyleneoctadecanoic acid was incubated with the pellet obtained from *T. pyriformis*. Four Erlenmeyer flasks of 25 ml were used for incubation in each experiment. The incubation medium was supplemented with 5.0 mM malonate.

The radioactive acetate was isolated as described earlier except that 10 μ mole of unlabelled acetate was added to each flask as carrier. The column fractions containing acetate from each experiment were pooled and dried under vacuum after the addition of 2.0 ml of 0.2 N ethanolic KOH. The samples collected from all experiments were pooled and dissolved in water to a total volume of 5.0 ml. An aliquot of 0.25 ml was transferred to a liquid scintillation vial and 0.25 ml of 2.5 N KOH was added to it. Fifteen ml Cab-O-Sil gel was then added and radioactivity was measured.

The rest of the sample was transferred to a 25 ml reaction flask and dried under vacuum on a rotary evaporator. Five mg of unlabelled sodium acetate was added and the sample was degraded by the Schmidt reaction (Phares, 1951) with some
modification (Dr. R. Heintz, personal communication) as follows:

The reaction flask was cooled on ice and 0.4 ml of 100% H₂SO₄ was added. The salt was dissolved by gently warming and shaking. The flask was placed again on ice and 50 mg sodium azide was added. The flask was shaken gently with warming until the sodium azide was nearly dissolved. A trap containing 3.0 ml of 5% KMnO₄ in 1.0 N H₂SO₄ was connected to the reaction flask and another one containing 3.0 ml of 0.3 N NaOH was connected to the first trap. The reaction flask was then placed in a water bath at 45 C and the temperature was increased to 70 C over a period of 20 minutes and maintained at that temperature for 45 minutes. CO₂-free air was then allowed to pass slowly through the system for 10 minutes. The traps were removed and the content of the reaction flask was made alkaline by the addition of 1.7 ml of 40% NaOH. Three ml of 5% KMnO₄ was then added and a new trap containing 3.0 ml of 0.3 N NaOH was connected to the reaction flask. The reaction flask was heated in a boiling water bath for 15 minutes then cooled to room temperature while passing CO₂-free air through the system. The air was then disconnected and 1.4 ml of 10.0 N H₂SO₄, 1.4 ml of saturated aqueous sodium acetate and 2.7 ml of HgCl₂ reagent (prepared from 10.0 g HgCl₂, 15.0 g NaCl and water up to 100 ml) were added to the reaction flask. The flask was then placed in the boiling water bath for 30 minutes while passing CO₂-free air through, then the trap was
removed.

The content of each of the traps that contained 3.0 ml of 0.3 N NaOH was divided into two equal parts. They were then transferred to four liquid scintillation vials and dried under vacuum in a rotary evaporator. One-half of an ml of water was added to each vial, followed by the addition of 15 ml of Cab-O-Sil scintillation gel. When data were expressed as dpm, counting efficiencies were determined by the addition of toluene-$^{14}$C as an internal standard.
RESULTS AND DISCUSSION

Several biological systems have been reported to be able to cleave a cyclopropane ring in some compounds. Hall et al. (1969) reported the ability of the protozoan Ochromonas ma. amensis to cleave the 9,19-cyclopropane ring of some phytosterols such as cycloartenol and 4-methylene cycloartenol to form poriferasterol. In plants, an enzyme from bramble tissues was reported (Heintz et al., 1972a) which can open the 9,19-cyclopropane ring of cycloeucalenol, converting it to obtusifoliol. 4,4-dimethyl sterols, cycloartenol and 24-methylene cycloartenol, were found to be very poor substrates for that enzyme (Heintz et al., 1972b). The fungus Fusarium oxysporum, Schlectendahl which is capable of growth utilizing cyclopropanecarboxylic acid as its only source of carbon was able to convert this compound to γ-hydroxybutyric acid (Schiller and Chung, 1970a).

It is known that higher animals are not capable of opening the cyclopropane ring of CFAs (Chung, 1966). However, they are capable of degradation of these acids from the carboxyl end to the ring (Wood and Reiser, 1965; Chung, 1966) which leads to the accumulation of CFAs with shorter chains. On the other hand, some protozoa that are known to feed on sources that contain high concentrations of CFAs, such as bacteria, contain no such short chain CFAs. Therefore, it is possible to assume that such protozoa are able to metabolize
4-9

the CFAs.

Metabolism of CFAs by *O. danica* Cells

At the beginning of this work, *O. danica* was chosen to study the biodegradation of CFAs. *O. danica*, which is a photosynthetic phytophlagellate protozoan, is capable of ingestion of particles, including bacteria (Pringsheim, 1952). At the stationary phase of growth, *E. coli* is known to contain a high concentration of $C_{17}$ (24%) and $C_{19}$ (24%) CFAs (Law et al., 1963). Therefore, *E. coli* cells were used as a source for the CFAs.

*O. danica* cells were grown in a mineral medium to which an *E. coli* culture, at the stationary phase of growth, was added. Samples were taken at the beginning of incubation and after 3 and 5 days of incubation. *O. danica* and *E. coli* cells were isolated from each sample by centrifugation. Cellular fatty acids were then isolated, methylated and identified by analytical GLC. Figures 2-4 show the fatty acid methyl ester pattern at the beginning of incubation and after 3 and 5 days of incubation. The fatty acid methyl esters with even carbon numbers (Figure 2) were identified using standard fatty acid methyl esters having even carbon numbers of 8-20. The $C_{19}$ CFA methyl ester was identified by comparison with standard dihydrosterculic acid methyl ester. The $C_{17}$ was identified as described by Ackman (1972). The peaks in Figures 3 and 4 that have the same retention time as those in Figure 2 are
Figure 2. Gas chromatogram of fatty acid methyl esters from *E. coli* and *O. danica* cells at the beginning of the incubation

Figure 3. Gas chromatogram of fatty acid methyl esters from *E. coli* and *O. danica* cells after 3 days of incubation

Figure 4. Gas chromatogram of fatty acid methyl ester from *E. coli* and *O. danica* cells after 5 days of incubation
for the same fatty acid methyl esters. The unlabelled peaks shown in the gas chromatograms are the unsaturated fatty acids preceding the saturated fatty acids of the same chain length. As can be seen from Figures 2-4, the cellular CFAs remained constant even after 5 days of incubation.

During incubation, the medium turned deep green in color indicating a healthy growth of the protozoan cells. Also, microscopic study revealed a large increase in protozoan cell number. The fatty acid pattern changed with time as indicated by the synthesis of new fatty acids accompanied by the increase or decrease in others (Figures 2-4). These changes indicate normal fatty acid synthesis in the *O. danica* cells but no detectable degradation of the CFAs.

Bacterial cells ingested by *O. danica* are digested and utilized, as indicated by the observation that the dietary biotin requirement can be satisfied by bound biotin in dead *Thiobacillus* cells (Aaronson and Baker, 1959). *O. danica* cells were harvested after 5 days of incubation with *E. coli* and washed twice. Microscopic study of a suspension of washed cells showed no *E. coli*. Figure 5 shows the fatty acid methyl esters of those washed *O. danica* cells. It shows the presence of C17 and C19 CFAs which are absent from the fatty acid methyl esters of protozoan cells grown in a chemically defined medium (Figure 6). This indicates the ingestion of *E. coli* cells by *O. danica*.

Magat and Tipton (1970) reported that when C17 and C19
Figure 5. Gas chromatogram of fatty acid methyl esters from *O. danica* grown on *E. coli* cells and then washed free of bacterial cells.

Figure 6. Gas chromatogram of fatty acid methyl esters from *O. danica* grown in a chemically defined medium.
CFAs labelled with $^{14}\text{C}$ at the methylene carbon were fed to *O. danica*, some of the label appeared as $^{14}\text{C}_2\text{O}_2$. The present work showed no decrease in the amount of those CFAs. This may be due to the relative intensity of the gas chromatographic method compared to the tracer method. Microscopic examination of the culture medium after 5 days indicated the presence of *E. coli* cells in large numbers.

Metabolism of CFAs by *T. pyriformis* Cells

In an attempt to find a better system to study the biodegradation of CFAs, another protozoan was used, *T. pyriformis*. When *T. pyriformis* cells were grown in an *E. coli* culture that was supplemented with minerals required by the protozoa, almost all *E. coli* cells disappeared from the medium in 5 days. The disappearance of *E. coli* cells from the culture medium was determined by microscopic examination of culture samples.

To determine whether the CFAs of *E. coli* cells were degraded completely by *T. pyriformis*, samples were taken from the culture at the beginning of incubation and after 3 and 5 days of incubation. Lipids were extracted from each sample, saponified and the free fatty acids were methylated and analyzed by GLC. The resulting chromatograms are shown in Figures 7-9. The peaks were identified as described before. There is a significant reduction in the amount of CFAs ($C_{17}$ and $C_{19}$) after 3 days (Figure 8) and only a very small amount of CFAs remained in the culture after 5 days of incubation.
Figure 7. Gas chromatogram of fatty acid methyl esters from a culture medium containing *E. coli* and *T. pyriformis* at the beginning of incubation.

Figure 8. Gas chromatogram of fatty acid methyl esters from a culture medium containing *E. coli* and *T. pyriformis* after 3 days of incubation.

Figure 9. Gas chromatogram of fatty acid methyl esters from a culture medium containing *E. coli* and *T. pyriformis* after 5 days of incubation.
DETECTOR RESPONSE
This reduction in the CFAs was not accompanied by any accumulation of shorter chain fatty acids with an odd carbon number, such as \( C_{11} \), as indicated by the absence of such a peak in the gas chromatograms (Figures 8 and 9). No fatty acid of an odd carbon number longer than 19 was detected. This indicates a complete biodegradation of the CFAs in E. coli by T. pyriformis.

To investigate the mechanism of their degradation by T. pyriformis, 3 flasks containing E. coli were prepared and a T. pyriformis culture was added to each of them as before. Pent-4-enoic acid at a final concentration of 0.1 mM and 1.0 mM was also added to 2 flasks at the same time as the T. pyriformis culture. The third flask was used as a control. It is known that pent-4-enoic acid (0.01 mM) strongly inhibits the oxidation of fatty acids in rat liver mitochondria (Senior et al., 1968). Holland et al. (1972) found that pent-4-enoyl-CoA was a good substrate for the acyl-CoA dehydrogenase and the product, penta-2,4-dienoyl-CoA, irreversibly inhibits the 3-oxoacyl-CoA thiolase.

Pent-4-enoic acid at a concentration of 0.1 mM and 1.0 mM was found to have no effect on fatty acid oxidation by T. pyriformis cells. The gas chromatograms indicated that the amount of all fatty acids after 4 days of incubation were decreased as compared to their amount at zero time. Also, the CFAs had almost completely disappeared. The fatty acid methyl ester profile of the control and of samples with pent-4-enoic acid were similar.
T. pyriformis is known to take propionate, isobytyrate and α-methyl-n-butyrate from the medium and incorporate them intact into longer chain fatty acids (Shorb, 1963). Long chain fatty acids can also be taken up by this organism (Lees and Korn, 1966). It is therefore most probable that pent-4-enoic acid was also taken up by this organism. It is possible that pent-4-enoic acid was detoxified by T. pyriformis cells or simply excreted.

Chemical Synthesis of CFAs

In order to isolate and identify the products of CFAs biodegradation and thence to find the mechanism of CFA oxidation, it was necessary to synthesize labelled CFAs. The isolation of the labelled products of their oxidation will also confirm the previous results that CFAs were completely degraded by T. pyriformis.

A CFA that contains a $^{14}$C labelled ring methylene carbon can be synthesized from $^{14}$CH$_2$I$_2$ and an unsaturated fatty acid methyl ester. However, labelled methylene iodide is not available commercially, but it can be prepared from acetone-1,3-$^{14}$C which is available. This can be done by converting acetone to iodoform which in turn can be reduced to methylene iodide. To investigate this possibility, methylene iodide was prepared from unlabelled acetone and used to react with methyl oleate to prepare cis-9,10-methylene-octadecanoate by the Simmons-Smith reaction.
As described in the experimental section, at the end of the Simmons-Smith reaction, the reaction products were purified by argentation TLC. When the spot corresponding to the CPA methyl ester standard was subjected to analytical GLC analysis, the gas chromatogram (Figure 10) indicated the presence of two compounds. The peak with the higher retention time had the same retention time as the standard \textit{cis}-9, 10-methyleneoctadecanoic acid methyl ester. Efforts to separate these two compounds by rechromatography using argentation TLC or reverse phase partition chromatography failed. Both of these compounds were then isolated separately by preparative GLC and subjected to analysis by mass spectroscopy. Each showed a molecular-ion peak at m/e 310, the molecular weight of a C\textsubscript{19} CPA methyl ester. Identical spectra were obtained for both compounds and these spectra in turn were identical to the spectra described by Wood and Reiser (1965) for \textit{trans}- and \textit{cis}-9,10-methyleneoctadecanoic acid methyl esters. Since the peak with the higher retention time shown in Figure 10 has a retention time identical to that of \textit{cis}-9,10-methyleneoctadecanoic acid methyl ester, the compound with the shorter retention time is probably \textit{trans}-9,10-methyleneoctadecanoic acid methyl ester. Simmons and Smith (1959) reported that the presence of excess methylene iodide and zinc-copper couple usually gave increased conversions of olefin to cyclopropane. On the other hand, Setser and Rabinovitch (1961) reported that though the Simmons-Smith
Figure 10. Gas chromatogram of trans- and cis-9,10-methyleneoctadecanonic acid methyl esters from the Simmons-Smith reaction
reaction is generally completely stereospecific giving cis-cyclopropanes from cis olefins, a small amount of isomerization to give the trans-product may occur in the presence of a large excess of the zinc-copper couple.

Two labelled CFAs were then chemically synthesized. \([11,12\text{-methylene-}^{14}\text{C}]\) cis-11,12-methyleneoctadecanoic acid was prepared from methyl vaccenate and acetone-1,3-\(^{14}\text{C}\). Two batches of this CFA were prepared and were found to have specific activities of 0.028 m Ci/m mole and 0.077 m Ci/m mole. The other CFA, cis-9,10-methyleneoctadecanoic acid with all carbons labelled with \(^{14}\text{C}\) except the methylene carbon, was prepared from oleic acid-U-\(^{14}\text{C}\) methyl ester and methylene iodide and had a specific activity of 0.15 m Ci/m mole.

**Metabolism of \([11,12\text{-methylene-}^{14}\text{C}]\) cis-11,12-methyleneoctadecanoic Acid by T. pyriformis cells**

For further determination whether the CFAs were oxidized beyond the ring by *T. pyriformis*, the isotope tracer technique was used. *T. pyriformis* cells were grown in the presence of \([11,12\text{-methylene-}^{14}\text{C}]\) cis-11,12-methyleneoctadecanoic acid. The radioactivity in CO\(_2\) evolved from the medium is shown in Figure 11. The results clearly indicate the ring could be opened and oxidized to CO\(_2\) by this organism.

*T. pyriformis* cells were also collected by centrifugation after 7 days of incubation with the labelled CFA. Lipids were extracted and glycogen was isolated from the cells. Glycogen
Figure 11. Oxidation of $[^{14}\text{C}]$ cis-11,12-methyleneoctadecanoic acid to $^{14}\text{CO}_2$ by T. pyriformis cells
is known to accumulate, under aerobic conditions, at the expense of intracellular lipids (Warnock and Eys, 1962). The radioactivities from the total culture were as follows:

- dpm in CO$_2$: 10407
- dpm in cellular chloroform-methanol extract: 38283
- dpm in cellular glycogen: 1585

No attempt was made to measure radioactivity in protein or in the culture medium. These results indicate that the CFA was not only oxidized to CO$_2$ by T. pyriformis, but also an oxidation intermediate was metabolized to glycogen.

A sizable portion of acetate-$^{14}$C was found by Hogg (1963) to be converted to glucose by this organism. The glyoxalate cycle enzymes, isocitrate lyase and malate synthase, are known to be present in T. pyriformis (Hogg, 1956). If acetate is a product of CFA metabolism, labelled glycogen could be produced via the glyoxalate cycle and gluconeogenesis.

In an attempt to isolate acetate as an intermediate in cyclopropane ring cleavage during CFA oxidation, an experiment was designed in which washed T. pyriformis cells were incubated with [11,12-methylene-$^{14}$C] cis-11,12-methyleneoctadecanoic acid in a buffered medium. The incubation was performed in the presence and absence of 10 mM malonate. The oxidation of the labelled CFA was observed by measuring the radioactivity in CO$_2$ released at the end of each incubation (Table 2). The radioactivity in the products of β-oxidation after separation on a silicic acid column was also measured.
Table 2. Oxidation of \([11,12\text{-methylene-}{^14C}]\text{cis-11,12-methyleneoctadecanoic acid}\) to \(^{14}\text{CO}_2\) and \(\beta\)-oxidation products \textit{in vivo}.

<table>
<thead>
<tr>
<th>Incubation medium(^a)</th>
<th>Incubation time (hr)</th>
<th>(^{14}\text{CO}_2)</th>
<th>Cpm Propionate-(^{14}\text{C})</th>
<th>Cpm Acetate-(^{14}\text{C})</th>
</tr>
</thead>
<tbody>
<tr>
<td>No malonate</td>
<td>2</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM malonate</td>
<td>2</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No malonate</td>
<td>4</td>
<td>64</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>10 mM malonate</td>
<td>4</td>
<td>54</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>No malonate</td>
<td>6</td>
<td>193</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>10 mM malonate</td>
<td>6</td>
<td>166</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

\(^a\)Each incubation flask contains \(36 \times 10^5\) cells and \(114,090\) dpm (0.077 m Ci/m mole) labelled substrate.
Titration of the fractions obtained from the silicic acid column chromatography of volatile fatty acids showed the appearance of carrier propionate primarily in fractions no. 12 and 13. Carrier acetate appeared in fractions no. 17-19. The radioactivity in CO$_2$ was not high. No label appeared in propionate and no significant label appeared in acetate.

**Fatty Acid Oxidation by *T. pyriformis* in vitro**

Since no significant label appeared in intermediates of [11-12, methylene-$^{14}$C] cis-11,12-methyleneoctadecanoic acid oxidation in vivo to allow further investigations, protozoan cells were homogenized and the 10,000 x g pellet was prepared. The two distinct layers which resulted from centrifugation of the cell homogenate were found to oxidize palmitic acid-U-$^{14}$C to $^{14}$CO$_2$. Kobayashi (1965) described the lower layer as the mitochondrial fraction and studied it for respiration and phosphorylation. In this study, the two layers were not separated and both were used.

Incubation with labelled substrates for all in vitro experiments were performed at 25 C for 4 hours with shaking. Each incubation flask contained the complete incubation medium for fatty acid oxidation mentioned in the experimental section unless otherwise specified.
Oxidation of palmitic acid-U-¹⁴C

Palmitic acid-U-¹⁴C was incubated with 20.4 mg pellet protein. The radioactivity found in ¹⁴CO₂ evolved at the end of incubation time is shown in Table 3.

In another preparation, the effect of malonate at two different concentrations was studied. The radioactivity in CO₂ evolved from the medium and acetate isolated from 3 incubation flasks are shown in Table 4.

The results shown in Tables 3 and 4 indicate that the pellet preparation was highly active in the oxidation of palmitic acid. Carnitine had no effect on the uptake and oxidation of palmitic acid (Table 3). This was not surprising as Wittles and Blum (1968) found that T. pyriformis has no free or bound carnitine and they could not detect any transferase activity. Malonate in both concentrations caused a large reduction in the amount of ¹⁴CO₂ evolved from the incubation medium (compare Tables 4 and 5). Some of the label that did not appear as ¹⁴CO₂ appeared in acetate. This indicates that β-oxidation was the mechanism by which palmitic acid was degraded. Fatty acid β-oxidation in a particulate preparation from T. pyriformis has not been reported previously.

The low yield of radioactivity in acetate compared to that in CO₂ may be due to the presence of the glyoxalate bypass enzymes in this organism (Hogg, 1956). These enzymes are found primarily in one of the particulate fractions, the
Table 3. Oxidation of palmitic acid-U-\(^{14}\text{C}\) to \(^{14}\text{CO}_2\) in vitro

<table>
<thead>
<tr>
<th>Incubation medium(^a)</th>
<th>(^{14}\text{CO}_2) (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>30830</td>
</tr>
<tr>
<td>Complete + 1.0 m M carnitine</td>
<td>29042</td>
</tr>
<tr>
<td>Complete but 5.0 m M NAD(^+)</td>
<td>28669</td>
</tr>
<tr>
<td>Complete but 1.25 m M NAD(^+)</td>
<td>26385</td>
</tr>
<tr>
<td>Complete but 2.0 m M ATP</td>
<td>29868</td>
</tr>
</tbody>
</table>

\(^a\)Each incubation flask contains 20.4 mg pellet protein and 240,000 dpm (0.06 m Ci/0.016 mg) labelled substrate.

Table 4. Oxidation of palmitic acid-U-\(^{14}\text{C}\) to \(^{14}\text{CO}_2\) and acetate-\(^{14}\text{C}\) in vitro

<table>
<thead>
<tr>
<th>Incubation medium(^a)</th>
<th>(^{14}\text{CO}_2) (cpm)</th>
<th>Acetate-(^{14}\text{C}) (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>Complete + 5 m M malonate</td>
<td>1295</td>
<td></td>
</tr>
<tr>
<td>Complete + 10 m M malonate</td>
<td>1327</td>
<td></td>
</tr>
<tr>
<td>Complete + 5 m M malonate</td>
<td>448</td>
<td></td>
</tr>
<tr>
<td>Complete + 10 m M malonate</td>
<td>428</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Each incubation flask contains 18.0 mg pellet protein and 240,000 dpm (0.05 m Ci/0.016 mg) labelled substrate.
peroxisosomes (Muller and Hogg, 1967). It has been reported also that when *T. pyriformis* is grown in a proteose peptone medium, the cells contain malate synthase in high activity but only small amounts of isocitrate lyase (Hogg and Kornberg, 1963; Reeves et al., 1961). When glucose is added to this medium, the formation of malate synthase is repressed.

To overcome the depletion of acetate resulting from $\beta$-oxidation by glyoxalate bypass, a pellet was prepared from *T. pyriformis* grown in a medium that contained 2% proteose peptone, 0.1% yeast extract and 1% glucose. A pellet prepared from *T. pyriformis* grown in the same medium but with no glucose was used as a control. Each pellet preparation was incubated with palmitic acid-$U^{-14}C$. The radioactivity which appeared in $CO_2$ and acetate is shown in Table 5. These results were not those expected. The pellet obtained from cells grown in the presence of glucose has much lower activity for oxidation of palmitic acid to both $CO_2$ in the absence of malonate and to acetate in its presence. Also, the ratio of radioactivity in acetate in the presence of malonate to that of $CO_2$ in its absence was about the same whether the cells were grown in the presence of absence of glucose. The low capability of the pellet obtained from cells grown in the presence of glucose to oxidize palmitic acid may be due to the repression of the enzymes involved in the oxidation of fatty acids as a result of the presence of glucose in the culture medium. In such a case, glucose may be the substrate that
Table 5. Oxidation of palmitic acid-U-\(^{14}\)C to \(^{14}\)CO\(_2\) and acetate-\(^{14}\)C by pellets prepared from \(T. \text{pyriformis}\) grown in the presence and absence of glucose

<table>
<thead>
<tr>
<th>Incubation medium(^a)</th>
<th>(^{14})CO(_2) (cpm)</th>
<th>Acetate-(^{14})C (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete(^b)</td>
<td>6591</td>
<td></td>
</tr>
<tr>
<td>Complete + 2.5 m M malonate(^b)</td>
<td>388</td>
<td>588</td>
</tr>
<tr>
<td>Complete + 5.0 m M malonate(^b)</td>
<td>253</td>
<td>480</td>
</tr>
<tr>
<td>Complete(^c)</td>
<td>24544</td>
<td></td>
</tr>
<tr>
<td>Complete + 2.5 m M malonate(^c)</td>
<td>813</td>
<td>1935</td>
</tr>
<tr>
<td>Complete + 5.0 m M malonate(^c)</td>
<td>452</td>
<td>1810</td>
</tr>
</tbody>
</table>

\(^a\)Each incubation flask contains 240,000 dpm (0.05 m Ci/0.016 mg) labelled substrate.

\(^b\)The pellet (20.3 mg protein) was prepared from cells grown in the presence of glucose.

\(^c\)The pellet (19.0 mg protein) was prepared from cells grown in the absence of glucose.

was used for energy and glycogen synthesis.

Oxidation of \([11,12\text{-methylene-}^{14}\text{C}] \text{cis-11,12-methyleneoctadecanoic acid}\)

Since the 10,000 x g pellet preparation prepared from \(T. \text{pyriformis}\) grown in the absence of glucose was found highly active in the oxidation of palmitic acid, it was used to study the oxidation of \([11,12\text{-methylene-}^{14}\text{C}] \text{cis-11,12-methyleneoctadecanoic acid}\) to both \(^{14}\)CO\(_2\) and \(\beta\)-oxidation products.
This CFA was incubated with the pellet preparation in the presence and absence of malonate. The radioactivity which appeared in CO$_2$ and in fatty acid $\beta$-oxidation products is shown in Table 6.

Table 6. Oxidation of [11,12-methylene-$^{14}$C] cis-11,12-methyleneoctadecanoic acid to $^{14}$CO$_2$ and $\beta$-oxidation products in vitro

<table>
<thead>
<tr>
<th>Incubation medium$^a$</th>
<th>$^{14}$CO$_2$</th>
<th>Propionate-$^{14}$C</th>
<th>Acetate-$^{14}$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>2008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete + 5 m M malonate</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Complete + 10 m M malonate</td>
<td>0</td>
<td>74</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Each incubation flask contains 13.9 mg pellet protein and 114,090 dpm (0.077 m Ci/m mole) labelled substrate.

The results clearly indicate that the CFA had been oxidized by the pellet preparation. Not only was the cyclopropane ring opened during oxidation to $^{14}$CO$_2$, but acetate was found as the product of the $\beta$-oxidation. The opening of the cyclopropane ring seems not to require any microsomal enzymes as the pellet was prepared by centrifugation at 10,000 x g, for 5 minutes, which is a low speed for
sedimentation of microsomes.

**Oxidation of cis-9,10-methyleneoctadecanoic acid labelled at all carbons with the exception of the methylene carbon**

Since cis-9,10-methyleneoctadecanoic acid has an odd number of carbons, we may be able to detect some propionate if it is completely degraded by β-oxidation. To investigate this possibility, cis-9,10-methyleneoctadecanoic acid labelled with $^{14}$C at all carbons with the exception of the methylene carbon was incubated with the 10,000 x g pellet preparation. Malonate and avidin were also added to the incubation medium as shown in Table 7. Table 7 also shows the radioactivity which appeared in CO$_2$ and in the fatty acid β-oxidation products.

These results indicate that in the presence or absence of avidin there was no significant radioactivity in propionate, though the CFA was oxidized well as indicated by the high production of $^{14}$CO$_2$ in the absence of malonate. Also, malonate caused a reduction in radioactivity in CO$_2$, but a high increase in radioactive acetate.

It is known that extracts of acetone-dried rat, ox or guinea pig liver mitochondria or whole mitochondria catalyze the carboxylation of propionate to succinate (Lardy and Adler, 1956; Friedberg et al., 1956). Preparations from biotin-deficient rats were much less effective than those of normal rats in catalyzing the carboxylation of propionate (Lardy and Adler, 1956). This metabolic defect was repaired by
Table 7. Oxidation of cis-9,10-methyleneoctadecanoic acid labelled with $^{14}$C at all carbons with the exception of the ring methylene carbon to $^{14}$CO$_2$ and fatty acid $\beta$-oxidation products

<table>
<thead>
<tr>
<th>Incubation medium$^a$</th>
<th>$^{14}$CO$_2$ cpm</th>
<th>Propionate-$^{14}$C cpm</th>
<th>Acetate-$^{14}$C cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>10098</td>
<td>3</td>
<td>49</td>
</tr>
<tr>
<td>Complete + 5 mM malonate</td>
<td>130</td>
<td>6</td>
<td>660</td>
</tr>
<tr>
<td>Complete + 100 $\mu$g avidin</td>
<td>10338</td>
<td>10</td>
<td>55</td>
</tr>
<tr>
<td>Complete + 200 $\mu$g avidin</td>
<td>9943</td>
<td>12</td>
<td>31</td>
</tr>
<tr>
<td>Complete + 100 $\mu$g avidin + 5 mM malonate</td>
<td>98</td>
<td>15</td>
<td>696</td>
</tr>
</tbody>
</table>

$^a$Each incubation flask contains 20.7 mg pellet protein and 168,070 dpm (0.15 m Ci/m mole) labelled substrate.

Injected biotin. This indicates that biotin is a necessary factor for the carboxylation of propionate. On the other hand, avidin was reported to bind biotin and prevented the carboxylation of acetyl-CoA required for fatty acid synthesis (Wakil and Gibson, 1960). The presence of avidin did not result in any significant amount of propionate from the CFA oxidation. This may be due to incomplete oxidation of the CFA which may lead to fragments intermediate in size between propionate and the long-chain fatty acids. It may also be due to depletion of the propionate by some other metabolic
pathway. In fact, very little is known about propionate metabolism by \textit{T. pyriformis}. Propionate was reported to double the rate of respiration when added to washed \textit{T. pyriformis} cells (Ryley, 1952). Also, an enzyme that converts \(\beta\)-hydroxypropionate to malonic semialdehyde is active in this organism (Den et al., 1959). \(\beta\)-hydroxypropionate may be produced from propionate through the intermediate acrylate.

**Mechanism of Oxidation of CFAs by \textit{T. pyriformis}**

As shown in Table 6, the cyclopropane ring of \([11,12\text{-methylene-}^{14}\text{C}]\ \text{cis-11,12-methyleneoctadecanoic acid}\) can be oxidized by the pellet preparation obtained from \textit{T. pyriformis} to both \(\text{CO}_2\) and acetate. The high radioactivity appearing in acetate as a result of oxidation of \text{cis-9,10-methyleneoctadecanoic acid} that was labelled at all carbons except the methylene carbon (Table 7), suggested that the CFA was degraded primarily by \(\beta\)-oxidation. However, the results obtained from those experiments do not answer the question of how the cyclopropane ring was opened. To answer this question \([11,12\text{-methylene-}^{14}\text{C}]\ \text{cis-11,12-methyleneoctadecanoic acid}\) was used. If the position of the label in acetate resulting from oxidation of this acid can be determined, then a mechanism for opening its cyclopropane ring could be postulated.

To investigate this possibility, several experiments were performed in which \([11,12\text{-methylene-}^{14}\text{C}]\ \text{cis-11,12-}
methyleneoctadecanoic acid was incubated with the 10,000 x g pellet preparation obtained from T. pyriformis cells and acetate was isolated. Column fractions containing acetate were pooled. Acetate was then degraded by the Schmidt reaction and radioactivity in each carbon was measured. The results were as follows:

<table>
<thead>
<tr>
<th>dpm in acetate degraded</th>
<th>1662</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpm in the first carbon</td>
<td>3</td>
</tr>
<tr>
<td>dpm in the second carbon</td>
<td>834</td>
</tr>
</tbody>
</table>

The results of the degradation indicate that essentially all the radioactivity in the acetate sample is in the methyl carbon.

Since only 50% of the original radioactivity in acetate appeared in the products, and to ensure that the first carbon of acetate did not, in fact, contain significant radioactivity, a standard acetate-1-\(^{14}\)C was degraded by the same method. Acetate-1-\(^{14}\)C (62 m Ci/m mole; Amersham) was diluted with 20 mg of unlabelled acetate before being degraded. The radioactivity in acetate-1-\(^{14}\)C and in the first and second carbon resulting from the degradation were as follows:

<table>
<thead>
<tr>
<th>dpm in acetate-1-(^{14})C degraded</th>
<th>7955</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpm in the first carbon</td>
<td>5562</td>
</tr>
<tr>
<td>dpm in the second carbon</td>
<td>90</td>
</tr>
</tbody>
</table>

The results indicate that degradation of acetate-1-\(^{14}\)C by the method used gives approximately a 70% yield of radioactivity from the first carbon and little carryover of radioactivity.
from the first carbon into degradation products of the second. All this indicates that cis-11,12-methyleneoctadecanoic acid labelled at the ring methylene carbon was degraded to acetate that was labelled at the second carbon.

A mechanism for the oxidation of \( [11,12\text{-methylene}^{14}\text{C}] \) cis-11,12-methyleneoctadecanoic acid by \( T. \text{pyriformis} \) is postulated as shown in Figure 12. This CFA is degraded to 3,4-methyleneoctadecanoic acid by oxidation. 3,4-methyleneoctadecanoic acid was found to accumulate when cis-9,10-methylenehexadecanoic acid was incubated with rat-liver mitochondria (Chung, 1966) and the ring methylene carbon of cis-9,10-methyleneoctadecanoic acid was not converted to CO\(_2\). The same author reported that cis-3,4-methyleneoctadecanoic acid was accumulated when cis-9,10-methyleneoctadecanoic acid was incubated with rat-liver mitochondria. The cis- and trans-3,4-methyleneoctadecanoic acids were also reported to accumulate in adipose tissue of rats fed cis- and trans-9,10-methyleneoctadecanoic acid (Wood and Reiser, 1965).

Since the \( ^{14}\text{C} \) labelled methylene carbon of cis-11,12-methyleneoctadecanoic acid appeared as \( ^{14}\text{CO}_2 \) and acetate-2-\( ^{14}\text{C} \) in this work, it is reasonable to assume that the cyclopropane ring was opened by the fatty acid \( \beta \)-oxidation system. The particulate fraction incubated with the labelled CFA in this work was prepared by centrifugation of \( T. \text{pyriformis} \) cells homogenate at a reasonably low speed for a short time. This will exclude the possibility of the need for microsomes.
Figure 12. Postulated mechanism of oxidation of \([11,12\text{-methylene}^{14}\text{C}]\) cis-11,12-methyleneoctadecanoic acid by \(T.\ pyriformis\).
for the cyclopropane ring cleavage. It is known that in higher animals many mixed-function oxidases occur associated with microsomes and are active in the hydroxylation of lipids and steroids and in the transformation of compounds foreign to the metabolic network (Mason et al., 1965). It is probable that the fatty acyl-CoA dehydrogenase of the β-oxidation system of T. pyriformis has a different specificity than that of higher animals, which makes it able to dehydrogenate the 3,4-methylenedecanoyl-CoA. This may be an adaptation of T. pyriformis to the utilization of bacterial cells as carbon sources. 2-enoyl-3,4-methylenedecanoyl-CoA will be the product of dehydrogenation of 3,4-methylenedecanoyl-CoA by the fatty acyl-CoA dehydrogenase. Once this compound is formed, it will then be hydrated by enoyl-CoA hydrase to 3-hydroxy-3,4-methylenedecanoyl-CoA. This compound can then easily tautomerise to 3-oxo-undecanoyl-CoA. DePuy (1968) reported that 2-phenyl-l-methyl-cyclopropanol readily isomerises to 4-phenyl-2-butanone under either acidic or basic conditions. 3-oxo-undecanoyl-CoA will then be further degraded by β-oxidation to propionyl CoA and acetyl-CoA.

In order to determine whether the cyclopropane ring was biodegradable and to establish a pathway for degradation, Schiller and Chung (1970a, b) studied the metabolism of cyclopropanecarboxylic acid (CCA) by the fungus Fusarium oxysporum. They found that the cyclopropane ring was opened by the fungus by direct addition of water across one of its carbon to carbon
single bonds, leading to the formation of γ-hydroxybutyric acid (GHB). The conversion proceeds through an intermediate compound, cyclopropanecarboxylate-X, that contains the intact cyclopropane ring. This intermediate compound can be converted to GHB by a cell-free extract. However, the cell-free extract cannot convert CCA to GHB. Also, the X-moiety is not CoA. In higher animals, 3,4-methylene-CFAs were the products of oxidation of 9,10-methylenehexadecanoic acid (Chung, 1966) and 9,10-methyleneoctadecanoic (Chung, 1966; Wood and Reiser, 1965). The present work suggests that T. pyriformis degrades CFAs primarily by β-oxidation. This also will lead to the formation of a 3,4-methylene CFA as an intermediate during oxidation. This compound has an isolated cyclopropane ring whereas the model compound used by Schiller and Chung (1970a, b) to study the degradation of the cyclopropane ring has the ring conjugated with a carboxyl group. This difference in structure would be expected to have a great effect on the reaction paths open to the two compounds. Also, a compound X was required for the cleavage of the cyclopropane ring of CCA which is not CoA indicating a system other than fatty acid β-oxidation was involved. In conclusion, the cleavage of the cyclopropane ring of CFAs by T. pyriformis is not analogous to the cleavage of CCA-cyclopropane ring by Fusarium oxysporum but is a rather simple adaptation of β-oxidation.
The purpose of this work was to study the metabolism of bacterial cyclopropane fatty acids (CFAs) by protozoa. Two protozoa, *Ochromonas danica* and *Tetrahymena pyriformis* were used. No degradation of CFAs was detected by gas-liquid chromatography when *E. coli* cells rich in CFAs were fed to *O. danica*. *T. pyriformis* cells were found to ingest all *E. coli* cells present in a culture medium in 5 days. This was accompanied with an almost complete disappearance of CFAs from the total medium. The disappearance of the bacterial $C_{17}$ and $C_{19}$ CFAs was not accompanied by the accumulation of shorter or longer CFAs.

For further investigation of the metabolism of CFAs by *T. pyriformis*, two labelled CFAs were chemically synthesized. $[11,12$-methylene-$^{14}$C] *cis*-11,12-methyleneoctadecanoic acid was prepared by Simmons-Smith reaction from $^{14}$C labelled methylene iodide and methyl vaccenate. Labelled methylene iodide was prepared by converting acetone-1,3-$^{14}$C to iodoform which in turn was reduced to methylene iodide. The other CFA, *cis*-9,10-methyleneoctadecanoic acid labelled with $^{14}$C at all carbons except the ring methylene carbon, was prepared from unlabelled methylene iodide and oleic acid-$U^{-14}$C methyl ester.

Growing *T. pyriformis* in a proteose peptone-yeast extract medium containing $[11,12$-methylene-$^{14}$C] *cis*-11,12-methyleneoctadecanoic acid resulted in the release of $^{14}$CO$_2$. 
indicating the ability of this organism to cleave the cyclo-
propane ring. The label also appeared in the cellular
glycogen.

A particulate fraction of cell homogenate (pellet) was
prepared from T. pyriformis cells and was found active in the
oxidation of palmitic acid-U-\textsuperscript{14}C to both CO\textsubscript{2} and acetate.
Carnitine had no effect on palmitic acid oxidation to CO\textsubscript{2}.
cis-9,10-methyleneoctadecanoic acid labelled at all carbons
except the ring methylene carbon was also oxidized to
labelled CO\textsubscript{2} and acetate by the pellet preparation. However,
no significant accumulation of propionate could be detected
even in the presence of avidin. When [11,12-methylene-\textsuperscript{14}C]
cis-11,12-methyleneoctadecanoic acid was incubated with the
pellet preparation, the label appeared in CO\textsubscript{2} in the absence
of malonate and in acetate in its presence. Degradation of
acetate by the Schmidt reaction indicated that only the
methylene carbon of acetate was labelled. A pathway for
biodegradation of bacterial CFAs by T. pyriformis by a modi-
fication of \textbeta-oxidation was proposed.
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