METABOLISM OF CYCLOPROPANE FATTY ACIDS BY OCHROMONAS DANICA

by

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TABLE OF CONTENTS

INTRODUCTION

Cyclopropane fatty acids, particularly lactobacillic acid (cis-11,12-methylene octadecanoic acid) and its C_{17} analog (cis-9,10-methylene hexadecanoic acid) occur in appreciable amounts in a number of bacterial species.

Their structures, chemical nature and the mechanisms involved in their biosynthesis have been studied in detail. However, their metabolism and function in bacterial cells are still unknown.

Studies in higher animals have suggested the degradation of cyclopropane fatty acids to the ring and the inability to convert the labeled methylene carbon of the cyclopropane fatty acids to carbon dioxide, resulting in the accumulation of shorter-chain cyclopropane fatty acids. No such accumulation has been detected in microorganisms indicating complete degradation of cyclopropane fatty acids in microorganisms feeding on bacteria which contain these acids.

Ochromonas danica, being a heterotrophic and phagotrophic biflagellated protozoan, feeds on bacteria and is likely to be able to metabolize the cyclopropane fatty acid contained in these organisms. Moreover, some investigations have indicated the metabolism of acetate and fatty acids by O. danica. Cultures of O. danica can be readily maintained on a chemically defined medium. For these reasons O. danica has been chosen for the study of the metabolism of cyclopropane fatty acids.

The objectives of this study are primarily to determine whether propionate is produced in the oxidation of cyclopropane fatty acids, to detect the intermediates of fatty acid metabolism in O. danica and to ascertain whether O. danica can cleave the cyclopropane rings of biosynthetically prepared labeled cyclopropane fatty acids.

REVIEW OF THE LITERATURE

Cyclopropane Fatty Acids

Cyclopropane fatty acids are unique both in their chemical nature and the reactions involved in their biosynthesis. Interest and research on this type of compounds began in 1950 with the isolation of lactobacillic acid (Hofmann and Lucas, 1950). In recent years other cyclopropane fatty acids have been shown to occur in a number of bacterial species and some plant oils. The structures of naturally occurring cyclopropane fatty acids are given in Table 1 (O'Leary, 1967).

Occurrence

Lactobacillic acid, named after the organism from which it was first isolated (Lactobacillus arabinosus), was the first fatty acid containing a cyclopropane ring found to be present in biological materials. It has now been characterized and has been shown to occur in appreciable amounts in many bacteria together with its C_{17} analog. The latter was originally detected in E. coli. Table 2 shows the distribution of C_{17} and C_{19} cyclopropane fatty acids in bacteria (Kates, 1964).

Shorter-chain cyclopropane fatty acids, C_{13} and C_{15}, have been shown to occur in C. butyricum lipids (Goldfine, 1968; Goldfine and Bloch, 1961).

Certain plant oils, those of the Malvaceae (Hibiscus syriacus and Sterculia foetida), contain cyclopropene acids,

Table 1. Cyclopropane fatty acids

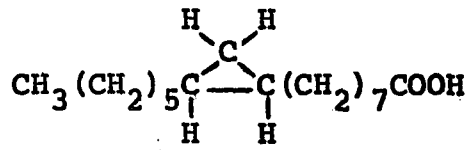
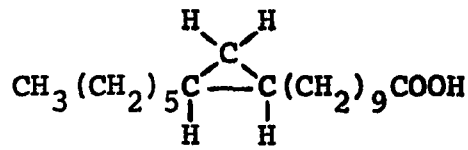
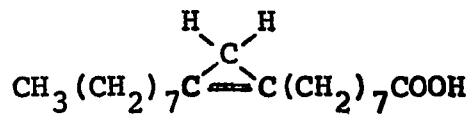
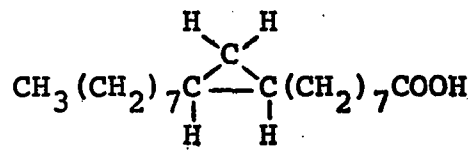
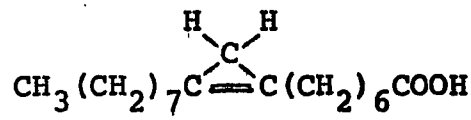
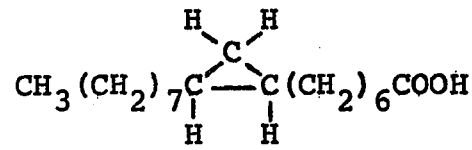
Common name	Systematic name	Structural formula
	cis-9,10-methylene hexadecanoic	
Lactobacillic	cis-11,12-methylene octadecanoic	
Sterculic	cis-9,10-methylene-9-octadecenoic	
Dihydrosterculic	cis-9,10-methylene octadecanoic	
Malvalic	8,9-methylene-8-heptadecenoic	
Dihydromalvalic	8,9-methylene heptadecanoic	

Table 2. Distribution of cyclopropane fatty acids (C_{17} and C_{19}) in various bacteria[a]

	17:Cy	19:Cy	References
Escherichia coli	22	7	Kaneshiro and Marr, 1961
	24	24	Law, 1961
Serratia marcescens	32	3	Bishop and Still, 1963
	44	9	Law et al., 1963
	27	10	Kates et al., 1964
Aerobacter aerogenes	25	6	O'Leary, 1962c
Agrobacterium tumefaciens	6	47	Kaneshiro and Marr, 1961
Lactobacillus plantarum	0	48	
Lactobacillus casei	0	49	Thorne and Kodicek, 1962
Lactobacillus acidophilus	0	28	
Streptococcus cremoris	-	45	
Streptococcus lactis	-	19	Mac Leod and Brown, 1963
Clostridium butyricum	9	5	Goldfine and Bloch, 1961

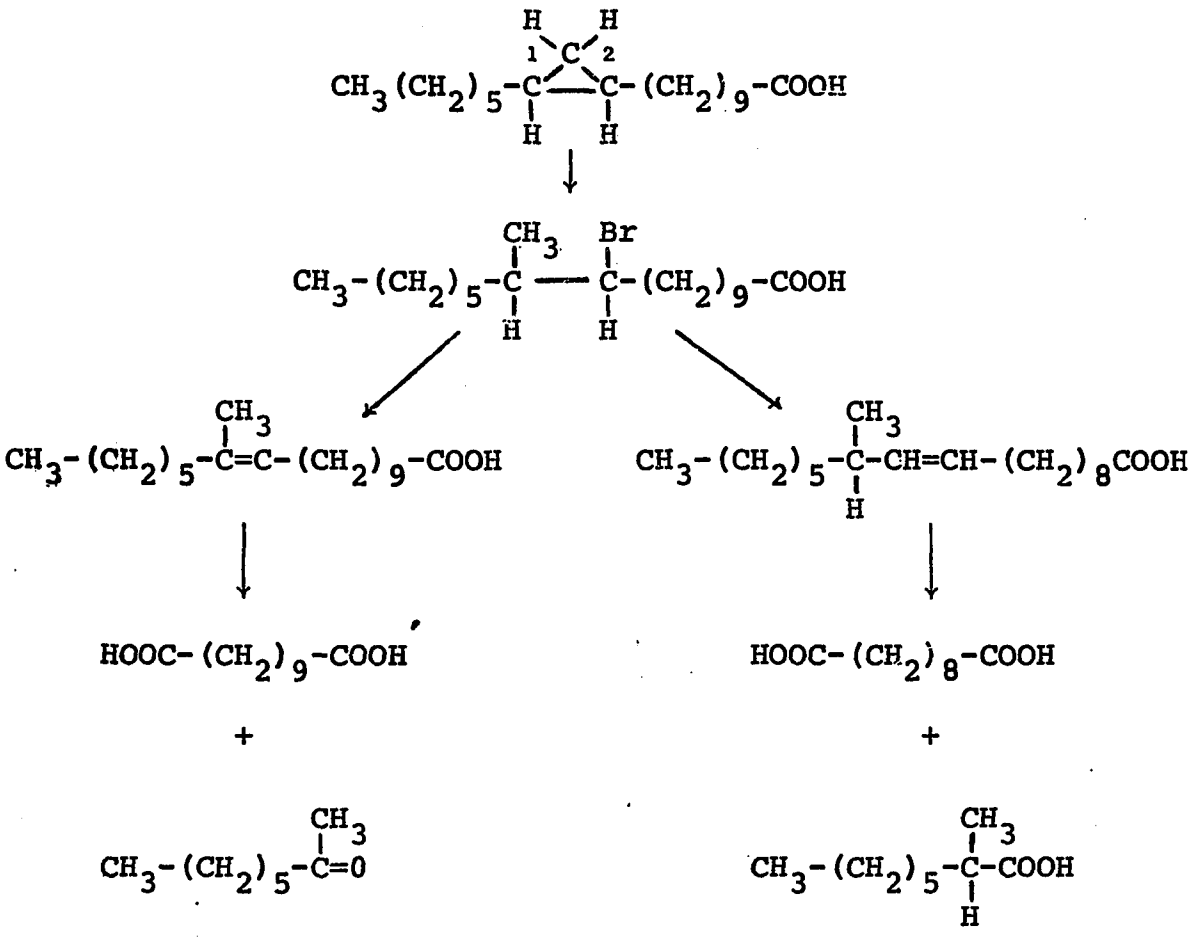
[a]Percentage of total fatty acid.

sterculic and malvalic acids as well as their cyclopropane analogues, dihydrosterculic and dihydromalvalic acids (Nunn, 1952; Hooper and Law, 1965; Johnson et al., 1967). Cyclopropene acids have not been encountered in true bacteria. However, O'Leary (1962b) has suggested the occurrence of a C_{15} cyclopropene acid together with C_{15} and C_{17} cyclopropane acids in pleuropneumonia-like organisms.

Meyer and Holz (1966) have recently demonstrated that seven species from four genera of zoomastigophoreans of the order Kinetoplastida (kinetoplastid flagellates) synthesize C_{19} cyclopropane acid, dihydrosterculic acid.

Chemical nature

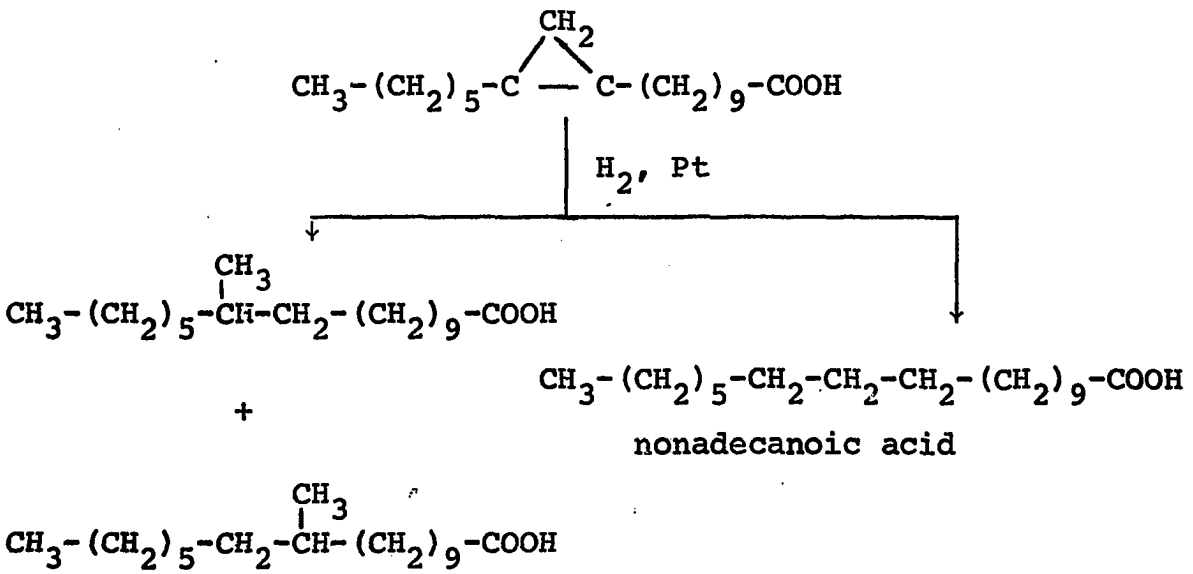
In the elucidation of the structure of lactobacillic acid, Hofmann and coworkers have demonstrated that this compound undergoes the characteristic reactions of the cyclopropane ring system. Lactobacillic acid was stable toward oxidation but was degraded by hydrogen bromide. Treatment of a cyclopropane fatty acid with hydrogen bromide followed by dehydrohalogenation and oxidation of the products, resulted in the formation of dibasic acids (Hofmann, Marco and Jeffrey, 1958). Further oxidation of the products with hypoiodite yielded iodoform. Hofmann and coworkers formulated the degradation pathway shown below (Hofmann, 1963).



In the above pathway, the ring cleavage occurred at position 2 and the methylene bridge carbon was separated from the rest of the chain. The nature of the products provided information regarding the position of cyclopropane ring in the original compound. Likewise, dihydrosterculic acid was degraded to azelaic acid ($HOOC(CH_2)_7COOH$) and suberic acid ($HOOC(CH_2)_6COOH$).

In the presence of hydrogen and platinum, cleavage of the carbon-carbon bonds in the cyclopropane ring occurs with the formation of a straight chain compound and a mixture of isomeric branched chain acids. The reaction involved in the

hydrogenolysis of lactobacillic acid is as follows (Hofmann, 1963).



(DL-11- and DL-12-methyloctadecanoic acids)

Naturally occurring Lactobacillic acid differs in melting point and diffraction pattern from chemically synthesized DL-cis-11,12-methylene octadecanoic acid (Hofmann, Orochena and Yoho, 1957). This non-identity of the natural and the synthetic products demonstrates that lactobacillic acid must be a distinct optical isomer (i.e., either D- or L-cis-11,12-methylene octadecanoic acid). Its optical rotation has not yet been determined. Optically active methyl octadecanoic acids are known to have extremely small optical rotation (Prout et al., 1948).

Biological activity

Long-chain mono- and poly-unsaturated fatty acids (C_{12} to C_{18}) are known to have the property of replacing biotin in the nutrition of certain bacteria. Cyclopropane fatty acids are the only group of compounds other than the unsaturated fatty acids demonstrated to have this growth-stimulating activity of biotin (Hofmann and Panos, 1954). With L. arabinosus and L. casei the position and stereochemistry of the cyclopropane ring have little effect on this activity (Hofmann, 1963). With L. delbrueckii, cis-vaccenic, dihydrosterculic and lactobacillic acids exert growth-promoting activity while the corresponding trans isomers have little effect or are completely inactive. Only cyclopropane fatty acids with greater than 11 carbon atoms exhibit this microbiological activity in the presence of suboptimal amounts of biotin (Hofmann and Panos, 1954).

Metabolism of Cyclopropane Fatty Acids

Biosynthesis

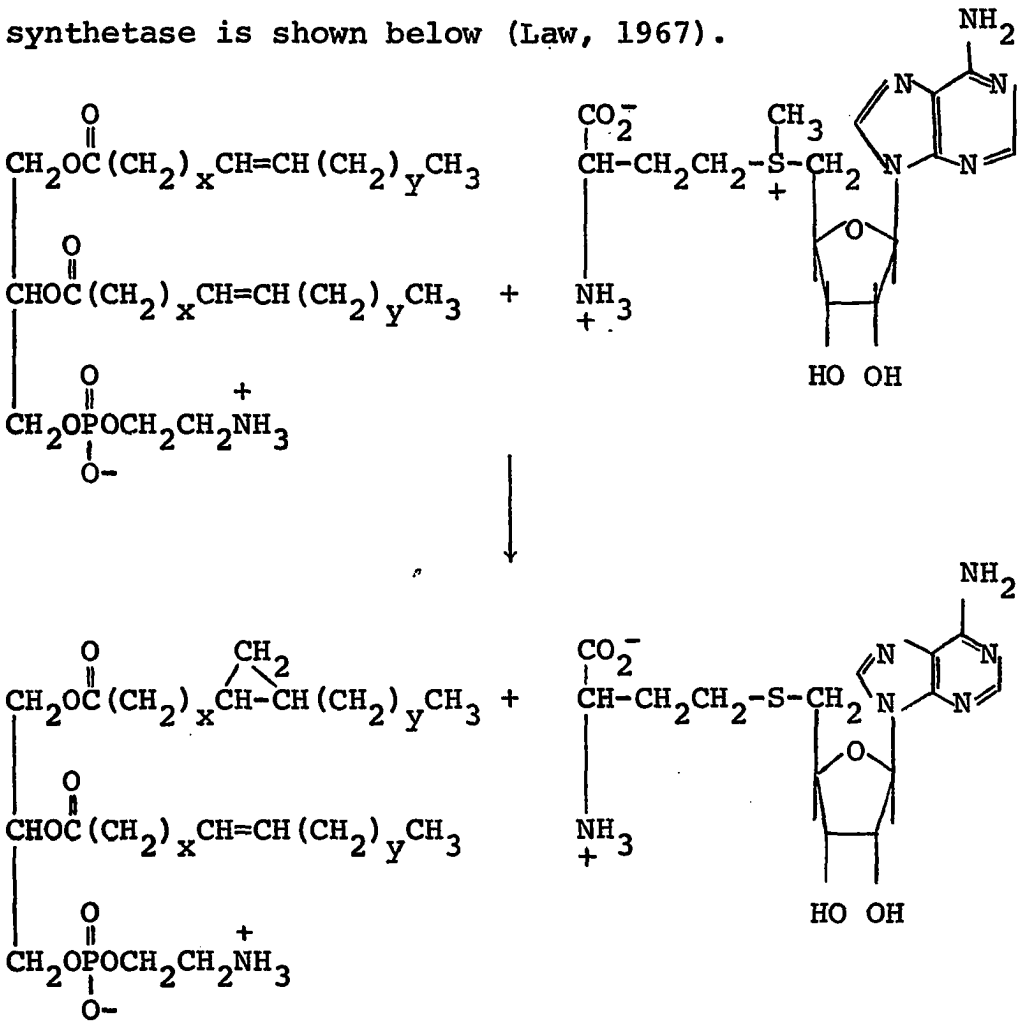
It is now well established that cyclopropane acids are formed by the insertion of a "C_1" fragment into the double bond of the corresponding olefinic acid. Experimental evidence on the nature of the monoenoic acid precursor for lactobacillic acid was obtained by employing cis-vaccenic acid-1-C^{14}. When the latter was added to actively growing cells, it was taken

into the cells unaltered, providing for 18 of the 19 carbon atoms of lactobacillic acid (O'Leary, 1959). The lactobacillic acid fraction of the lipids isolated contained 92% of the radioactivity (Hofmann and Liu, 1960). Addition of labeled oleic acid to a culture of L. arabinosus also produced labeled lactobacillic acid (O'Leary, 1959). It was suggested that oleic acid had undergone isomerization forming cis-vaccenic acid and lactobacillic acid. It has been demonstrated that methylation of the double bonds in long-chain fatty acids occurs when the bonds are 5 and 7 methylene groups removed from the methyl end in the formation of cyclopropane fatty acids (Scheuerbrandt et al., 1961).

O'Leary has shown that methionine is the source of the 1-carbon unit in the formation of the cyclopropane ring. This observation was later confirmed by Liu and Hofmann (1960), Chalk and Kodicek (1961) and Zalkin et al. (1963). The reaction was subsequently shown to be catalyzed by a soluble enzyme system from crude extracts of C. butyricum and S. marcescens (Chung and Law, 1964; Zalkin et al., 1963). Methionine which has been activated to S-adenosylmethionine was the methyl donor. The enzyme, cyclopropane fatty acid synthetase, exhibits an absolute requirement for phospholipid containing an unsaturated fatty acid (Zalkin et al., 1963). Furthermore, the physical state of the phospholipid was observed to be a critical factor for the efficacy of the substrate. Crude

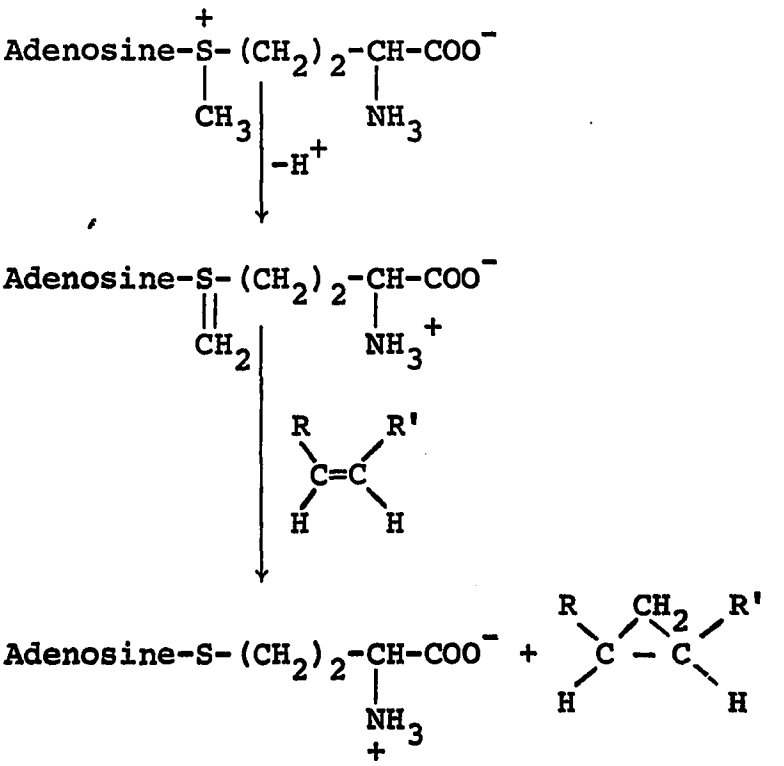
phosphatidyl ethanolamine dispersed in a clear micellar suspension was an excellent substrate. Purified phosphatidyl ethanolamine was not only a poor substrate but was also found to be an inhibitor of the methylene group incorporation from S-adenosyl-methionine into phosphatidyl ethanolamine (Chung and Law, 1964). Lipids which do not form micellar solutions were ineffective as substrates. According to Bangham and Dawson (1959), the surface charge on the phospholipid micelles is required for the enzyme-micelle interaction. Dispersion into a clear micellar suspension is therefore necessary for the effectiveness of the substrate. The surface charge can be altered by ionic surfactants, metal cations and anions. Anionic surfactants stimulate cyclopropane synthetase activity while cationic and neutral surfactants were inhibitory (Chung and Law, 1964). Other unsaturated phospholipids such as phosphatidyl glycerol or phosphatidic acid served as substrates in the presence of Ca^{++} (Thomas and Law, 1966). The enzyme was also shown to be specific for fatty acids in the β or γ positions of the phospholipid. In this connection, Hildebrand and Law (1964) have shown that in the phospholipids of several bacteria, the unsaturated fatty acids were predominantly in the β position with the exception of C. butyricum phospholipid in which the unsaturated fatty acids were mostly in the γ position. The distribution of cyclopropane fatty acid was determined by that of the olefinic fatty acids.

The overall reaction catalyzed by cyclopropane fatty acid synthetase is shown below (Law, 1967).



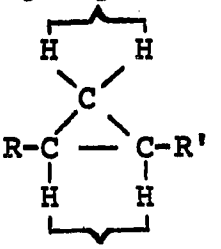
Studies on the mechanism of cyclopropane ring formation with trideuteromethyl-methionine have established that the methionine methyl group is transferred along with two of its hydrogen atoms to the olefinic fatty acid chain (Pohl et al., 1963). Furthermore Polacheck et al. (1966) have shown that the vinyl hydrogens of the unsaturated acid precursor were retained. Oleic acid 9,10-d_2 was converted into dideuterated dihydrosterculic acid by L. arabinosus. These observations therefore

indicated that intermediates having a double bond on carbon atoms 9 or 10 (in the case of dihydrosterculic acid formation), after the addition of the methylene carbon from methionine, could not have been involved. A hypothetical reaction for the addition of the methylene group of S-adenosyl methionine to the unsaturated fatty acid chain is shown below (Pohl et al., 1963).



The origins of all protons in the cyclopropane ring is as follows (Polacheck et al., 1966):

from methyl group of methionine



from olefinic acid

Johnson et al. (1967) have studied the biosynthesis of cyclopropane and cyclopropene fatty acids in the seeds and fruits of the plant Malvaceae. There was an incorporation of methyl ^{14}C methionine into dihydrosterculic and sterculic acids. This agrees with the observations of Hooper and Law (1965). The methylene addition across the double bond is analogous to that described in bacteria. The initial product was dihydro sterculic acid since about 93% of the radioactivity was incorporated into this compound after a four hr. incubation period and the percentage of label decreased within 24 hrs, with an increase in the radioactivity of sterculic acid. This indicated a desaturation of dihydrosterculic acid to sterculic acid. Dihydromalvalic acid may be formed by either α oxidation of dihydrosterculic acid or by the methylene addition to a C_{17} monounsaturated acid (Johnson et al., 1967). These investigators postulated the reactions involved in the biosynthesis of cyclopropene fatty acids shown below:

oleic acid + $\cdot CH_2$ → dihydrosterculic acid → dihydromalvalic
↓ ↓
stearolic acid + $\cdot CH_2$ → sterculic acid → malvalic
↑
C_{17} monounsaturated acid + $\cdot CH_2$ → dihydromalvalic acid

Excellent reviews on the biosynthesis of cyclopropane fatty acids are available: Hofmann (1963), Goldfine (1968), Law (1967) and O'Leary (1962a, 1967).

Degradation

Most of the studies on cyclopropane fatty acids have concentrated on the mechanism of the biosynthesis of the cyclopropane ring. There is at present very little known about the degradation or oxidation of these acids, their function in the microorganisms containing them as well as their effect, if any, on the normal fatty acid metabolism.

Wood and Reiser (1965) have demonstrated that in rats, synthetic cis- and trans-9,10-methylene octadecanoate were degraded by oxidation up to the cyclopropane ring. The rest of the chain was not catabolized. When these cyclopropane acids were fed to rats, cis- and trans-3,4-methylene dodecanoate accumulated in the adipose tissues. The normal fatty acid metabolism was, however, not affected. Similar results were obtained with rat liver mitochondria (Chung, 1966). Cis-9,10-methylene hexadecanoic and cis-9,10-methylene octadecanoic acids were synthesized with S-adenosyl-(methyl ^{14}C)-methionine and cyclopropane fatty acid synthetase from C. butyricum. The cyclopropane acids were incubated with rat liver mitochondria and the carbon dioxide released was collected. The labeled methylene carbon of the cyclopropane acids was not converted to carbon dioxide, suggesting that the cyclopropane ring was not oxidized. C_{11} and C_{13} cyclopropane fatty acids accumulated in the incubation mixture. Whether cyclopropane fatty acids are oxidized in a similar manner in microrganisms is not known.

Metabolic role of cyclopropane fatty acids

Although the cyclopropane acids are present in appreciable amounts in many bacterial species, there is as yet no known function of these compounds in bacterial cells.

It has been established that cyclopropane fatty acids promote growth of microorganisms in the presence of suboptimal amounts of biotin (Hofmann et al., 1957, 1959). Law et al. (1963) have suggested that this biological property of cyclopropane acids is due probably to the cell's requirement for a phospholipid to fulfill a structural function and that this phospholipid acquires the properties for carrying out such function in the presence of unsaturated and cyclopropane fatty acids. This is supported by the finding that soluble phospholipid micelles can be prepared only with phospholipids containing unsaturated fatty acids or saturated fatty acids with cyclopropane acids, but not with phospholipids containing only saturated fatty acids (Fleischer and Klouwen, 1961). The structural role of cyclopropane acids is suggested by the following findings: these acids are present largely as phospholipids (Kaneshiro and Marr, 1962; Law, Zalkin and Kaneshiro, 1963); phosphatidylethanolamine, the main fraction of bacterial lipids undergoes no detectable metabolic turnover of the phosphorus moiety (Kanfer and Kennedy, 1963); the formation of morphological variants in E. coli is correlated with the inhibition of cyclopropane fatty acid synthesis (Weinbaum and Panos, 1966).

Their apparent indispensibility in the organisms containing them is suggested by the finding that in bacteria containing lactobacillic acid, the amounts of other major fatty acids may be changed or eliminated, but it was not possible to reduce below a certain level the amount of lactobacillic acid (Hofmann et al., 1957).

Two hypotheses have been proposed by O'Leary (1967) on the role of cyclopropane fatty acids. Due to the chemical lability of the ring carbon of cyclopropane acids, they were suggested to function as carrier or storage forms of 1- carbon units. Since these acids were formed during the late logarithmic and early stationary growth phases, it was suggested that their formation was only a case of an aging phenomenon or detoxifying activity for the removal of undesirable products such as S-adenosyl methionine or unsaturated fatty acids.

Membranes of bacteria contain unsaturated fatty acids which are susceptible to oxidation by oxygen or free radicals. The conversion of the unsaturated acids into cyclopropane acids could be a means of saturating these acids thereby preventing lipid peroxidation and retaining some physical properties (e.g., low melting point) of unsaturated fatty acid. Bacteria are not known to contain any lipid antioxidants (Law et al., 1963).

Ochromonas danica

Characteristics

The order Chrysomonadida consists of fresh water, soil and marine flagellates. Two closely related species, O. danica and O. malhamensis were obtained in pure culture by Pringsheim (1952) and were described as single celled, biflagellated algae which are phototrophic, heterotrophic and phagotrophic. They grow both on high molecular weight dissolved food and low molecular weight chemically defined media (Hutner, et al., 1953), in the light or in the dark. Their growth requirements were investigated by Hutner and his colleagues (1953). It was found that there were two absolute substrate requirements: sugar or glycerol and any one of the citric acid cycle intermediates. O. malhamensis differs from O. danica in that it does not grow in the absence of carbohydrate even in the light. This is due to an insufficient amount of chlorophyll in the chromatophore (Myers, 1951). Both O. danica and O. malhamensis contain only chlorophyll a (Lwoff, 1964). However, O. danica has 6 times more chlorophyll than O. malhamensis, accounting for the feeble photosynthesis in the latter (Myers, 1951; Myers and Graham, 1956). O. danica, unlike O. malhamensis, exhibits no requirement for vitamin B_{12}. However, it also requires biotin and thiamine. Biotin may be obtained by ingestion of bacterial cells. Chrysomonads, in general, exhibit properties of both animals and plants; they are able to ingest particulate

food and they undergo photosynthesis. Chrysomonads are considered to have the simplest nutritional requirement of any 'animal' studied (Hutner et al., 1953). They are therefore excellent tools in the study of biochemical systems of animals as well as plants and lower organisms.

Lipid composition

The lipid composition of O. danica varies with the culture conditions. When grown in the dark in a chemically defined medium containing no lipid, the fatty acid composition was shown to be as follows (Haines et al., 1962):

	percent
myristic	15
palmitic	10
stearic	3
oleic	8
linoleic	16
linolenic	2
γ-linolenic	10
arachidic	1
8,11,14-eicosatrienoic	5
arachidonic	11
docosapentaenoic	2-4

More recently, a sulfolipid, identified as 1,14-docosyldisulfate has been isolated from O. danica (Mayers and Haines,

1967). This compound has been demonstrated to occur in a wide variety of microorganisms, from Pseudomonas to Tetrahymena pyriformis (Haines, 1965). It is the third sulfolipid to be characterized. The other two are cerebroside sulfate and the chloroplast sulfonolipid 6-sulfoguinovosyl-diglyceride. Furthermore, it is the only long-chain alkyl sulfate found in nature.

A heterogeneous class of chlorosulfolipids has been found to occur in O. danica (Elovson and Drysdale, 1969). One was identified as 13-chlorodocosane-1,14-diol disulfate. Similar compounds containing two to six chlorine atoms were also detected. Chlorination of these sulfolipids occurs mostly during the stationary phase. The chlorosulfolipids were reported to have an inhibitory effect on the fatty acid synthetase in O. danica.

Metabolism

Reazin (1954) has investigated the metabolism of acetate and palmitate by O. malhamensis. Both substrates were metabolized by starved or non-starved cells. There was complete oxidation of acetate. Palmitate produced a slight stimulation of cell respiration. Pre-incubation of the cells with palmitate for an hour resulted in a higher respiration rate as compared to those which were not previously incubated. The formation of adaptive enzymes was suggested.

Studies on the oxidation of carboxy labeled acetate, formate and propionate in the presence of vitamin B_{12} by O.

malhamensis (Arnstein and White, 1962) have shown that vitamin B_{12} stimulated propionate oxidation, as determined by measurement of the radioactivity of the CO_2 evolved. The oxidation of formate and acetate were only slightly affected or not at all. Moreover, with vitamin B_{12} deficient cells, there was an almost complete block of propionate oxidation. It has been reported that when vitamin B_{12} was absent, the isomerization of methyl malonate to succinate was inhibited (Marchesi and Lajtha, 1961). Vitamin B_{12} also stimulated the growth of O. malhamensis (Arnstein and White, 1962).

O. danica exhibited a response to acetate and propionate similar to that of O. malhamensis (Sabo, 1966). In the absence of vitamin B_{12} there was a high rate of oxygen uptake when acetate was the substrate, while a lower than endogenous rate resulted from propionate. Addition of vitamin B_{12} caused an increase in the oxygen uptake of cells incubated with propionate. The metabolism of long-chain fatty acids by O. danica was also investigated (Sabo, 1966). Palmitate produced a slightly higher than endogenous respiration rate. This rate was lowered by vitamin B_{12}. There was a slight stimulation of oxygen consumption of cells in the presence of heptadecanoic and lactobacillic acids. However, this appeared to be within experimental error. The uptake of oxygen was further increased by vitamin B_{12}. The effect of vitamin B_{12} in the presence of synthetic cis-9,10-methylene octadecanoic acid was less than

with lactobacillic acid. The intermediate products of the metabolism of cis-9,10-methylene octadecanoic acids were investigated. The CoA esters were isolated from O. danica cells and converted to hydroxamates. The latter were detected by thin-layer chromatography. In the absence of vitamin B_{12}, acetate was identified as the intermediate while with added vitamin B_{12} an unidentified compound having a higher R_f than propionate was detected.

EXPERIMENTAL PROCEDURE

Equipment

Gas-liquid chromatographic analyses were carried out with an Aerograph model 1520 gas chromatograph equipped with a flame ionization detector and matrix temperature programmer. The glass column measured 6 ft. in length and 1/8 in. in diameter. It was packed with 10% OV-1 coated on chromosorb Q (100-200 mesh) obtained from Applied Science Laboratories, State College, Penn. The carrier gas was nitrogen with a flow rate of 30 ml. per minute. Hydrogen has the same flow rate as nitrogen. The preparative gas chromatograph used for the isolation of the cyclopropane fatty acids from E. coli was a modified Wheelco gas chromatograph equipped with a thermal conductivity detector and a column (1/4 in. x 6 ft.) packed with 15% (w/w) ethylene glycol succinate on Chromosorb P, 60/80 mesh (Anachrom Laboratories). The temperature of the column was maintained at 180°C with a helium gas flow of 38 ml. per minute.

The mass spectrometer used was a Perkin-Elmer model 270 equipped with a chromatographic inlet system and a Honeywell high speed recorder. The glass column of the chromatographic inlet system was packed with 10% OV-1 on Chromosorb Q (100-200 mesh), also a product of Applied Science Laboratories.

For isotope counting two instruments were used; a Nuclear-Chicago automatic planchet counter with model 470 gas

flow detector and data print out facilities and an Ansitron II liquid scintillation counter equipped with a refrigeration unit.

Reagents

Vitamin B_{12} was obtained from Sigma Chemical Co., St. Louis, Mo. The BF_3-butanol reagent is a product of Applied Science Laboratories, State College, Penn. Methionine methyl-^{14}C having a specific activity of 52 mC/mmole was purchased from International Chemical and Nuclear Corp., Amar Rd., City of Industry, Calif. Thin-layer chromatographic standards, acetohydroxamate and propionylhydroxamate, were prepared according to the procedure of Lipmann (1945). Bray's reagent for radioactivity measurements with the scintillation counter was prepared as described by Bray (1960). Hexane, absolute methanol and chloroform were redistilled and the purity determined by gas chromatography. All other reagents used were reagent grade and were not purified further before use.

Materials

Substrates for growth curves

Sodium acetate (reagent grade) was obtained from Mallinckrodt Chemical Works, St. Louis, Mo. and was used without further purification.

Sodium propionate was purchased from Sigma Chemical Co., St. Louis Mo. Characterization and determination of purity

was done by gas-liquid chromatographic analysis of butyl propionate. The butyl ester was prepared following the method of Jones and Davidson (1965) with BF_3-butanol solution obtained from Applied Science Laboratories, State College, Penn. The mixture was heated in a steam bath for 10 minutes, excess butanol was evaporated off by means of a rotary evaporator and the extract taken up with hexane for gas-liquid chromatographic analysis. A reference mixture consisting of the butyl esters of the fatty acids of butter was used. Sodium propionate appeared to contain some acetate. The gas chromatographic analysis showed four small unidentified extraneous peaks aside from the propionate peak. Sodium propionate was used without further purification.

Heptadecanoic and palmitic acids were obtained from Applied Science Laboratories, State College, Penn. The test of purity was carried out by gas-liquid chromatography. Methylation of the acids consisted of the treatment of 1-2 mg. of the acids in methylation flasks[1] with 2 ml. of redistilled absolute methanol containing 2% H_2SO_4 at 55°C for 2 hours (Selvey, 1960). One ml. of water was then added into each flask and the flask was agitated for about 10 seconds. Hexane was added, followed by enough water to bring the solution to the neck of the flask,

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[1]Made by ISU Chemistry Glass Shop. These were patterned after the reaction flasks available from Microchemical Specialities Co., Berkeley, California.

shaking after each addition. The hexane solutions were injected into the gas chromatograph. The column temperature was maintained at 180°C. The standard used was a mixture of the methyl esters of the fatty acids of butter. The gas-liquid chromatographic analysis of heptadecanoate and palmitate showed the materials to be pure. Each gave a single peak.

Synthetic cis-9,10-methylene octadecanoic acid was obtained from Analabs, Hamden, Conn. The purity of the acid was determined by gas-liquid chromatography of the methyl ester following the procedure described for heptadecanoic and palmitic acids (above). Cis-9,10-methylene octadecanoic acid appeared pure. The gas chromatogram showed only one peak.

Valeric acid was a product of Matheson, Coleman and Bell, Norwood, Ohio. The undiluted acid was thrice crystallized in an acetone-dry ice bath. Upon gas-liquid chromatography of the purified sample, three extraneous peaks appeared which were insignificant in size compared to the valeric acid peak.

^{14}C-labelled substrates for oxidation experiments

Sodium acetate-1-^{14}C was purchased from Isotope Specialties, Inc. No further purification was carried out.

Stearic acid-1-^{14}C from Fischer Scientific, Fair Lawns, N. J., was methylated with methanol containing 2% H_2SO_4 as described in the preceding section. Gas-liquid chromatography of methyl stearate at a column temperature of 200°C gave a single peak.

Methyl oleate-2-^{14}C was obtained from Applied Science Laboratories, State College, Penn. The ester in hexane solution was chromatographed to determine its purity. Only one peak appeared on the gas chromatogram at a column temperature of 200°C.

Oleic acid-1-^{14}C was prepared from methyl oleate-2-^{14}C by saponification of 1 ml of the latter with 1 N NaOH at 37°C for one hour. The mixture was then acidified with 6N HCl, extracted with ether and the solvent evaporated off under vacuum.

Biosynthesis of the cyclopropane fatty acids by E. coli was carried out with the following conditions and procedures: Growth conditions for E. coli.

The stock culture of E. coli (BF) was obtained from Dr. J. Horowitz, Iowa State University. The cells were grown on nutrient agar slants for about 48 hours before they were transferred to the synthetic growth medium specified by Davis and Mingioli (1950). The composition of the growth medium is as follows:

	gm. per l.
K_2HPO_4	7.0
KH_2PO_4	3.0
Sodium citrate·3 H_2O	0.5
$MgSO_4 \cdot 7\ H_2O$	0.1
$(NH_4)_2SO_4$	1.0

Glucose	2.0
H_2O	q.s. 1 l.

Glucose and $MgSO_4 \cdot 7\ H_2O$ were sterilized separately and added to the sterile growth medium prior to inoculation with E. coli.

The cells were grown in 250 ml. Erlenmeyer flasks containing 100 ml. of growth medium in a New Brunswick gyratory shaker at 37°C overnight. Flasks containing 500 ml. of growth medium were then inoculated with the cells (5% v/v). After growing the cells at 37°C overnight another cell transfer was made into 30 l. of growth medium contained in fermenters which were previously equilibrated at 37°C with a constant temperature bath. The cells were harvested after 47 hours by centrifuging at 18,000 rpm with a Lourdes betafuge model A-2 continuous flow centrifuge.

For the biosynthesis of cyclopropane fatty acids labeled in the methylene carbon of the cyclopropane ring, the E. coli cells were transferred from agar slants to synthetic growth medium as previously described. The flasks containing 100 ml. each of growth medium were inoculated each with 5 ml. of cells grown overnight in the growth medium. The flasks were incubated at 37°C in a Dubnoff metabolic shaking incubator for 2 hours before 10 μC. of methionine methyl-^{14}C in 2 ml. of water was added to each flask. The incubation period was 24 hours. The cells were harvested by centrifugation at 16,000 rpm for 10 minutes in a Sorvall refrigerated centrifuge. The packed cells were resuspended in $CHCl_3$-CH_3OH (2:1 v/v).

Extraction of the fatty acids from E. coli The fatty acids were isolated from the packed E. coli cells according to the method of Law, Zalkin and Kaneshiro (1963) with some modifications. The procedure is outlined in Figure 1. The methyl esters of the fatty acids were prepared with methanol containing 2% H_2SO_4 as described earlier, then dissolved in hexane for analyses. During the preparation of labeled fatty acids both the unsaponifiable and saponifiable fractions were dissolved in ethyl ether and diluted to a total volume of 1 ml. One hundred µl. aliquots were placed on glass planchets and air-dried. Measurements of radioactivity was performed with a Nuclear-Chicago gas flow counter. The samples were counted for 10 minutes in duplicate, then redissolved in ether and added to the original solutions. The solvent was evaporated off under vacuum.

Analysis of the fatty acids from E. coli

Gas-liquid chromatographic analysis The total fatty acid fraction isolated from E. coli was methylated with absolute methanol containing 2% H_2SO_4 following the procedure described previously. The methyl esters of the constituent fatty acids were separated by preparative gas-liquid chromatography. The C_{17} and C_{19} cyclopropane fatty acid esters were collected in glass U-tubes immersed in liquid nitrogen. A mixture of the methyl esters prepared from butter served as a

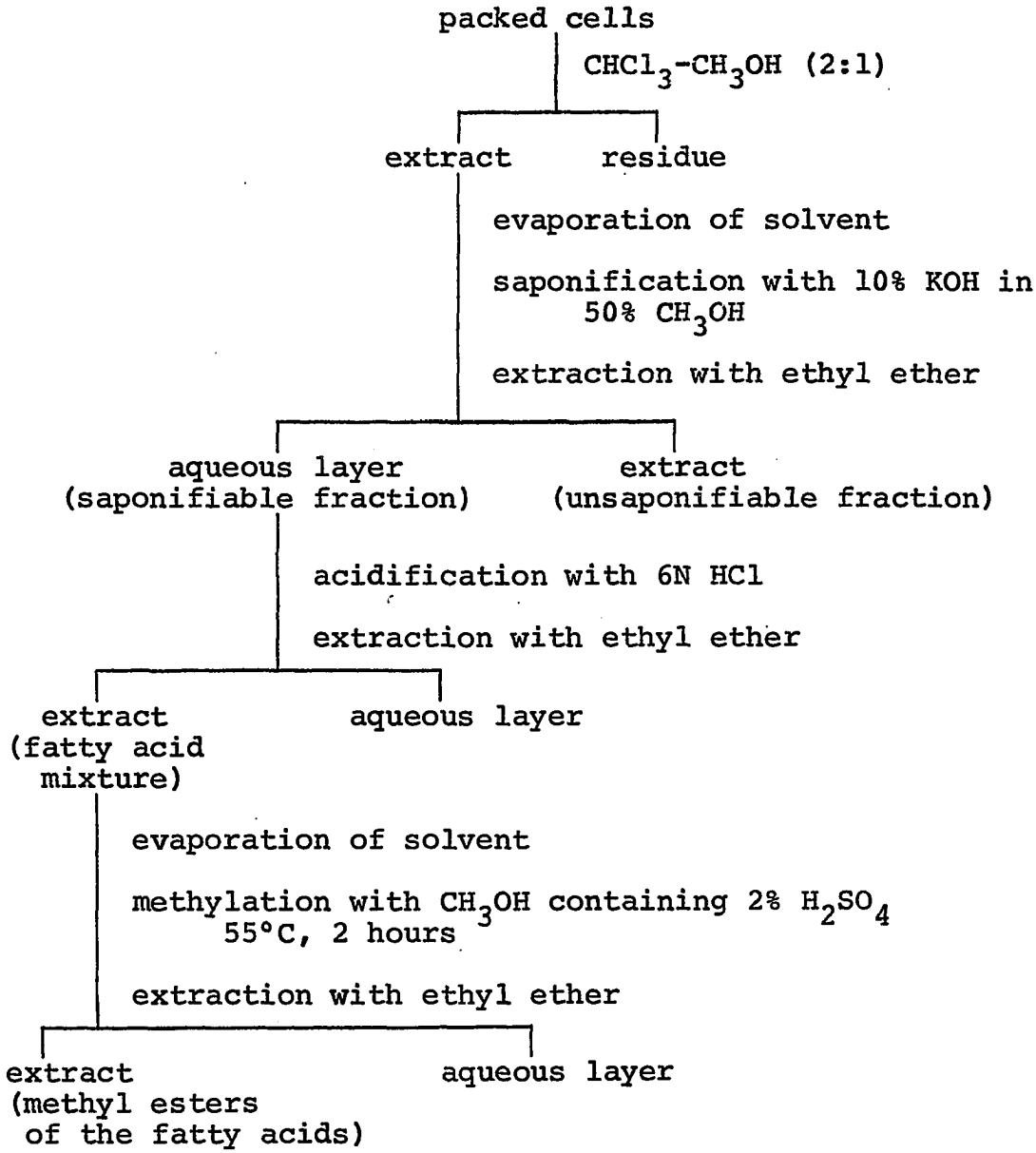


Figure 1. Isolation of the fatty acids from E. coli

standard. The labeled cyclopropane fatty acid esters collected in U-tubes were diluted with hexane to a total volume of 1 ml. Aliquots of 100 µl. were placed in glass planchets for the measurement of radioactivity with Nuclear-Chicago gas flow counter. The samples were counted for 5 minutes in duplicate, then redissolved in hexane and added to the original solutions. Hexane was evaporated off under vacuum

Mass spectrometric analysis The methyl esters of the fatty acid mixture were dissolved in hexane prior to analysis on a Perkin-Elmer mass spectrometer model 270. The column temperature was kept at 200°C.

Ochromonas danica

The Ochromonas danica stock culture was obtained from Haskins Laboratory, New York, N. Y. The culture was maintained by inoculation of 100 ml. fresh growth medium with 5 ml. of a 3 to 4-day-old stock culture. The composition of the growth medium according to Aaronson and Baker (1959) is shown in Table 3. The medium was obtained as a dry mixture from General Biochemicals, Inc., Chagrin Falls, Ohio. After dissolution it was sterilized in an autoclave for 15 minutes at 15 psi and 121°C. The cells were grown at room temperature in the light.

Table 3. The growth and mineral media used in the study of the metabolism of O. danica

Chemicals	Growth	Mineral
Nitrilotriacetic acid	0.02 g.	0.02 g.
KH_2PO_4	0.03 g.	0.03 g.
$CaCO_3$	0.0005 g.	0.0005 g.
$MgCO_3$ (basic)	0.04 g.	0.04 g.
$MgSO_4 \cdot 7\ H_2O$	0.1 g.	0.1 g.
NH_4Cl	0.005 g.	0.005 g.
Metals mixture[a]	1.0 mg.	1.0 mg.
Biotin	1.0 mg.	1.0 mg.
Thiamine HCl	0.1 mg.	0.1 mg.
L-Arginine HCl	0.04 g	
Glucose	1.0 g.	
L-Glutamic acid	1.0 g.	
Glycine	0.01 g.	
L-Histidine HCl	0.04 g.	
Distilled water, q.s., 100 ml.		
pH 5.0		

[a] $Fe(NH_4)_2SO_4 \cdot 6H_2O$, 0.2 mg.; $ZnSO_4 \cdot 7H_2O$, 0.1 mg.; $CuSO_4 \cdot 5H_2O$, 0.008 mg.; $CoSO_4 \cdot 7H_2O$, 0.01 mg.; H_2BO_3, 0.01 mg.; $MnSO_4 \cdot H_2O$, 0.05 mg.; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.005 mg.; $Na_2VO_4 \cdot 16\ H_2O$, 0.001 mg.

Growth Curves

Sodium acetate, sodium propionate and valeric acid were added to the growth medium as aqueous solutions before sterilization while heptadecanoic acid, palmitic acid and dl-cis-9, 10-methylene octadecanoic acid were added as stable suspensions to the sterile growth medium. These suspensions were prepared by injecting ethanol solutions of the fatty acids into hot distilled water in a flask and shaking the mixture during the addition, according to the method of Miller and Johnson (1960). The concentration of the acids in the suspensions was kept at approximately 1 mg. per ml. The suspensions were sterilized separately from the medium at 15 psi and 121°C for 15 minutes. The final concentration of the substrates in the growth medium was 0.001 M.

The cells from the stock culture of O. danica were isolated by centrifuging for 5 minutes in a clinical centrifuge and resuspending in a volume of sterile water equal to that of the original stock culture. Flasks each containing 100 ml. of the sterile growth medium and substrates were inoculated with 5 ml. of the cell suspension. Incubation of the cells was carried out in a constant temperature bath kept at 30°C. The growth of the cells was followed by taking 1 ml. samples from each flask at the start of the incubation period and at regular intervals afterwards. Collection of the samples was done with sterile pipettes. One ml. of the cells was fixed with a

solution having the following composition: ethanol: 72 ml.; formalin: 10 ml.; glacial acetic acid: 5 ml.; and water: 13 ml. The cell counts were taken with a hemocytometer and reported as number of cells per ml. The growth curves were drawn by plotting the cell counts against the incubation period in hours.

Identification of Some Components of the "Bound Lipids" from *O. danica*

Extraction and esterification of the "bound lipids"

The "bound lipids" were isolated according to the procedure outlined in Figure 2. The extraction method with boiling isopropanol followed by isopropanol-$CHCl_3$ (1:1 v/v) was described by Kates and Eberhardt (1957). The butylation procedure was that of Jones and Davidson (1965) using BF_3-butanol. A modified procedure of isolating the lipids was performed with cells that had been previously grown in normal growth medium. Performic acid treatment was substituted for alkaline hydrolysis for the CoA ester analysis. Formic acid and 30% hydrogen peroxide (45:5 v/v Hirs, 1956) was added to the residue after isopropanol $CHCl_3$ extraction. The reaction mixture was placed in ice and the reaction allowed to continue for 15 minutes. The unreacted formic acid was removed by titration of the mixture with 0.1 N $KMnO_4$ under alkaline condition. The ether extract was backtitrated with water. The rest of the procedure is shown in Figure 2 was followed.

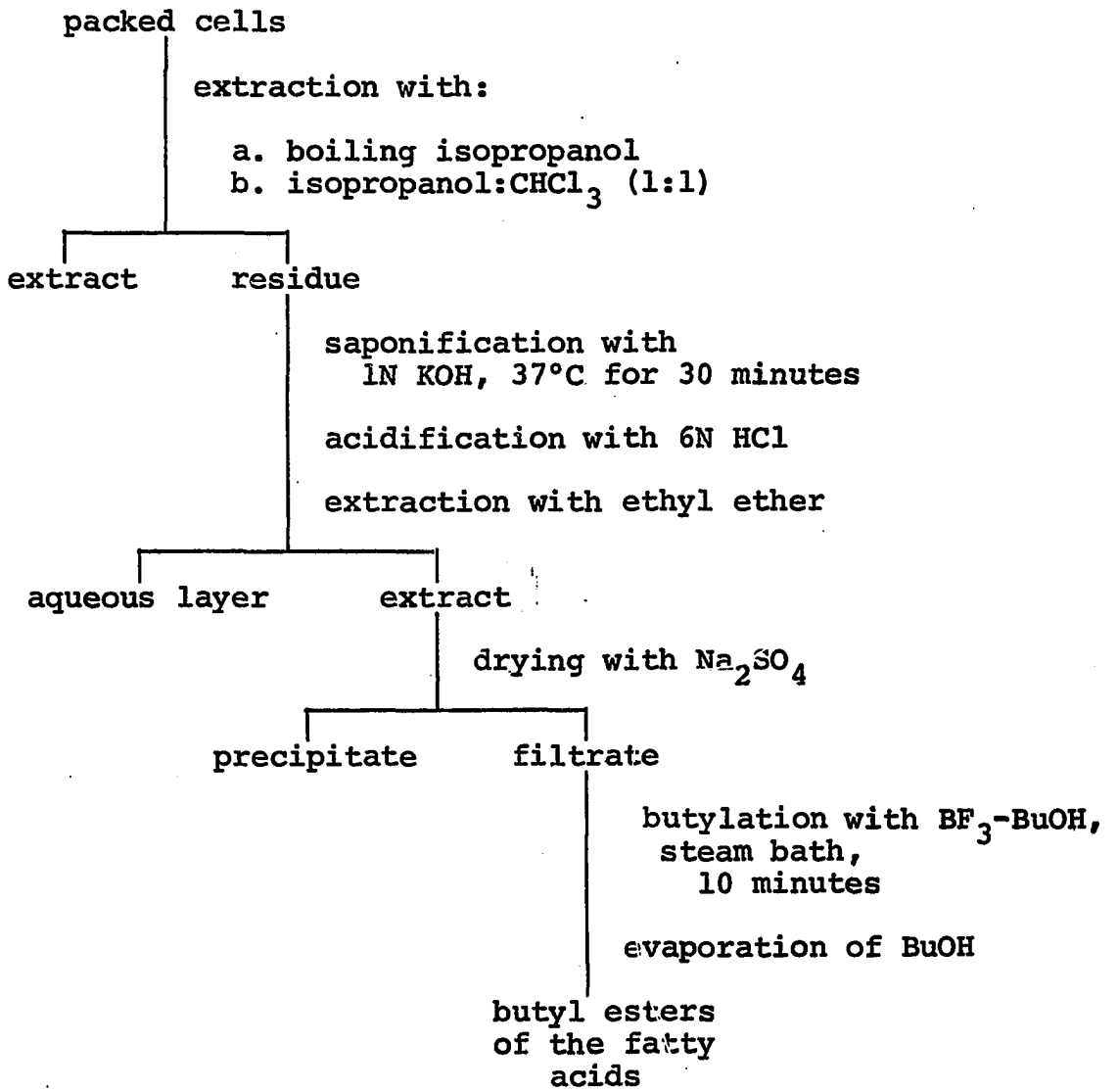


Figure 2. Isolation of "bound" lipids from O. danica

In one experiment, O. danica cells were incubated with heptadecanoic acid. The packed cells obtained after centrifugation of the stock culture were resuspended with an equal volume of sterile mineral medium. The composition of the medium is shown in Table 3 (Sabo, 1966). The cells were broken by sonication at 40% full power for one minute with a Bronwill Biosonik sonicator. Heptadecanoic acid suspension was added to the broken cells to a final concentration of 0.001 M. The mixture was incubated for an hour at room temperature.

Identification of the "bound acids" from O. danica

Gas-liquid chromatographic analysis The analysis was run isothermally at 75°C and at 200°C. The standard used consisted of a mixture of the butyl esters prepared from butter. The extraction of the fatty acids from butter was done with $CHCl_3$-CH_3OH (2:1 v/v) and the butylation with BF_3-butanol reagent.

Mass spectrometric analysis The butyl esters were dissolved in hexane. The analysis was run with increasing temperature from 90°C to 250°C. The column packing was 2.5% SE-30 on Chromosorb Q.

Thin-layer chromatographic analysis of the fatty acid hydroxamates The butyl esters from O. danica were converted into hydroxamates according to the method of Horning, (1964), using 3M neutral hydroxylamine in ethanol. This solution was prepared from hydroxylamine-HCl neutralized with KOH in ethanol.

The reaction was allowed to continue for 30 minutes. The ethanol was evaporated off and the hydroxamates were redissolved in hexane and applied on thin-layer plates coated with Silica gel G purchased from Applied Science Laboratories. The solvent systems used were: 1. water-saturated ether:CH_3OH, 9:1 v/v (Sabo, 1966); 2. water-saturated butanol (Stadtman and Barker, 1950); 3. Toluene:CH_3OH, 8:2 and 6:4 v/v (Trans, 1967). The standards used were acetohydroxamate and propionylhydroxamate prepared with the same procedure as the samples.

Attempt to assay acetate and propionate activation

Propionate activating enzyme activity was assayed by two methods. The first procedure was according to Jencks (1962) using hydroxylamine as acyl acceptor and measuring the pyrophosphate released as non-adsorbable, acid-labile phosphate.

$$RCOO^- + ATP + NH_2OH \rightarrow AMP + PP + RCONHOH$$

The O. danica cell suspension was prepared by centrifuging 200 ml. cells in growth medium with a clinical centrifuge and resuspending in 10 ml. of 0.1 M potassium phosphate buffer. The cells in an ice bath were sonicated at 40% full power with a Bronwill Biosonik sonicator, then centrifuged in a clinical centrifuge for 30 minutes in the cold. The supernatant was used for the assay.

The following reagents were added to each tube: 0.1 ml. 1 M Tris buffer, pH 8; 0.05 ml. 0.1 M ATP; 0.07 ml. 0.1 M

$MgCl_2$; 0.08 ml. 0.5 M NaF; 0.05 ml. 0.1 M glutathione, neutralized to pH 4; 0.1 ml. 1.85 M hydroxylamine and 0.1 ml. 0.05 M acetate or propionate. Enough water was added to make the final volume 1 ml. The tubes were equilibrated in a 37°C water bath for 5 minutes before 0.2 ml. of cell suspension was added to each tube. Incubation was continued for 30 minutes, after which the reaction was stopped with 0.5 ml. of 10% (w/v) TCA. The tubes were then centrifuged in a clinical centrifuge for 10 minutes, 1 ml. aliquots were taken and added to 4 ml. each of NaAc. Norit charcoal was added, the contents of the tubes were stirred and centrifuged. One ml. aliquots of the supernatant were removed for the determination of inorganic and acid-labile phosphate by the Fiske-Subbarow method (1925). Blank tubes to which TCA solutions were added before the cell suspensions were run with the samples.

The second assay method (Berg, 1960) involved the determination of the rate of formation of aceto- or propionylhydroxamate with CoA as acyl acceptor in the presence of ATP and substrates. To each tube are placed the following reagents: 0.1 ml. 0.1M potassium phosphate buffer, pH 7.5; 0.1 ml. 0.1 M ATP, 0.02 ml. 0.005 M CoA; 0.05 ml. 0.1 M $MgCl_2$; 0.1 ml. 0.5 M NaF; 0.05 ml. 0.2 M glutathione; 0.1 ml. 0.2 M hydroxylamine, pH 6.5 and 0.1 ml. 0.1 M acetate or propionate. The procedure was the same as that described in the first method except that incubation was at 37°C for 20 minutes and the reaction was

stopped by the addition of 2 ml. of 0.37 M $FeCl_3$ containing 0.2 M TCA and 0.66 M HCl. After centrifugation in a clinical centrifuge for 10 minutes the optical densities of the supernatant fluids were measured with a Beckmann DU spectrophotometer at 540 mu. The blank, for endogenous substances reacting with hydroxylamine, contained no CoA.

Oxidation of ^{14}C Labeled Acids

Incubation procedure

Sodium acetate-1-^{14}C was added as a water solution while the other substrates were added as suspensions. These suspensions were prepared in the same manner as the substrates used in the growth curve determinations (Miller and Johnson, 1960). The Warburg flasks and the suspensions were sterilized in an autoclave at 15 psi and 121°C for 15 minutes before incubation of the substrates with the O. danica cells. Each flask contained the radioactive substrate and 3-4 x 10^8 cells in the main compartment. In the center well was placed 0.2 ml. or 0.3 ml. of 20% KOH and the side arm contained 0.4 ml. of the acid used to stop the reaction (6N H_2SO_4 or 10% TCA). Incubation was carried out in a Dubnoff shaking incubator at 30°C. Incubation was varied from 2 to 48 hours depending upon the substrates. The incubation was terminated by the addition of the acid from the side arm of the flask into the main compartment. The flasks were then placed in ice. In the control flasks, the acid was added immediately after the addition of the substrates

and the flasks were kept in ice. At the end of the incubation period, the flasks were stored at about 4°C for at least 18 hours before the measurement of the radioactivity in order to collect additional $^{14}CO_2$.

Measurement of the amount of ^{14}C in CO_2 and ^{14}C remaining in the incubation mixture

One hundred µl aliquots were taken from the KOH solution containing the absorbed $^{14}CO_2$, in the center well of the Warburg flasks, and placed in scintillation vials. Five ml. of scintillation fluid (Bray's solution) was added to each vial. Radioactivity measurements were made with the Ansitron II scintillation counter. Samples were counted for 4 minutes each, in duplicate. Since the mixture of the KOH solution with Bray's reagent gave a pale pink color, a correction factor for the difference in counts from the actual counts with a colorless solution was determined by measuring the radioactivity of a standard fatty acid in Bray's solution, before and after the addition of 100 µl. of 20% KOH. The correction factor calculated was 1.09. The counts obtained were then multiplied by this factor.

The ^{14}C remaining in the incubation mixture contained in the main compartment of the Warburg flasks was determined by taking 0.5 ml. aliquots, adding this to 5 ml. of Bray's reagent in the scintillation vials and counting the isotope with the Ansitron II scintillation counter. The mixture of the samples

with Bray's solution gave a green color due to pigments from the cells. To correct for the effect of the pigment on the counting efficiency, 0.5 ml. of the cell suspension in mineral medium was added to 5 ml. of Bray's solution with a standard fatty acid. The radioactivity was measured before and after the addition of the cell suspension. The counts of the samples were corrected with the calculated correction factor (1.40).

In one experiment with oleic acid-1-^{14}C, the Nuclear-Chicago gas flow counter was used. Ten µl. aliquots of the KOH solution and incubation mixture were applied on glass planchets and allowed to dry under an infra-red lamp before counting. The samples were counted for 10 minutes each, in duplicate.

Determination of the respiration of O. danica in the presence of H_2SO_4 and TCA

One hundred ml. of cells in normal growth medium was centrifuged in a clinical centrifuge for 10 minutes then resuspended in 100 ml. of mineral medium. This medium was sterilized by filtration through a sterile millipore filter. The cells were allowed to remain in the mineral medium for 2 days in the dark. After this period, the cells were again centrifuged and resuspended in 10 ml. of fresh mineral medium. One ml. of the cell suspension was placed in each Warburg flask. A small piece of fluted filter paper was fitted in the center well of the flask which contained 0.2 ml. of 20% KOH solution. Into the side arm of the flask was added 0.4 ml. of the acid

(6N H_2SO_4 or 10% TCA). Respiration of the cells was measured with a Gibson respirometer. The bath was maintained at 25°C. Readings were taken at regular time intervals (5, 10 and 20 minutes) before and after (10, 30, 60 and 120 minutes) the addition of the acids. Control flasks to which no acid was added, were run simultaneously with the other flasks.

RESULTS AND DISCUSSION

In higher animals the degradation of cyclopropane fatty acids to the ring is suggested by the accumulation of shorter chain cyclopropane acids (Wood and Reiser, 1965; Chung, 1966) and the inability to convert the labeled ring carbon of the cyclopropane acids to carbon dioxide (Chung, 1966). No short chain cyclopropane acids similar to those found to accumulate in higher animals have been detected in microorganisms. These observations, coupled with the fact that cyclopropane fatty acids are present in high concentration in the organisms producing them, suggest that the cyclopropane ring is degraded completely by microorganisms feeding on bacteria which contain these acids.

O. danica has been chosen for the study of the mechanism of cyclopropane acids metabolism for the following reasons: 1) O. danica is both heterotrophic and phagotrophic. a) It is known to feed on bacteria containing cyclopropane fatty acids and is likely to be able to metabolize these acids. Studies by Sabo (1966) have indicated metabolism of acetate and fatty acids by O. danica. b) It can be grown readily in both light and dark conditions and on a chemically defined medium (Aaronson and Baker, 1959). 2) Propionate metabolism by O. malhamensis, a closely related species to O. danica has been demonstrated to be enhanced by vitamin B_{12} (Arnstein and White, 1962). Since the cyclopropane fatty acids are odd-

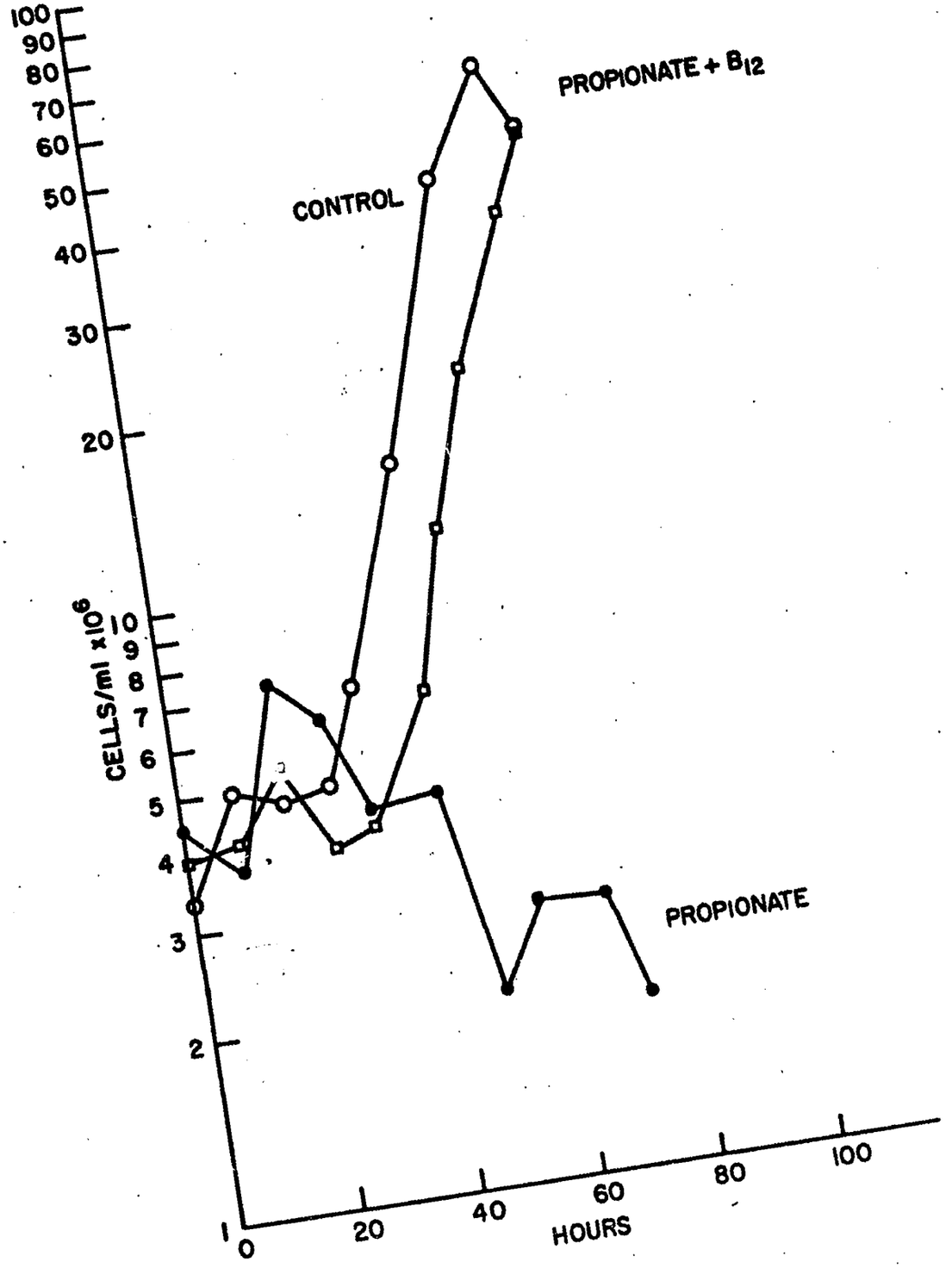
carbon compounds, it was thought that propionate might be a product of this degradation.

Metabolism Studies

The growth of O. danica in the presence of propionate has been studied. The growth curve (Figure 3) shows that propionate causes an inhibition of growth of the organism. This inhibition is reversed by vitamin B_{12}. Arnstein and White (1962) have demonstrated that vitamin B_{12}-deficient O. malhamensis cells are unable to oxidize propionate due to a block in the conversion of methyl malonate into succinate. Addition of vitamin B_{12} produced a stimulation of propionate oxidation. However, comparison of the effects of vitamin B_{12} analogues on the oxidation of propionate and the growth of the organism showed no definite correlation. It was concluded that this effect of vitamin B_{12} on the oxidation of propionate was not related to the stimulation of growth of O. malhamensis cells by vitamin B_{12}.

The effect of propionate and vitamin B_{12} on the growth of O. danica was determined several times (Table 8). It was found that the extent of inhibition of growth with propionate as well as the enhancement of growth with the addition of vitamin B_{12} varied. The cell cultures which were less inhibited, however, showed no bacterial contamination upon examination with an oil emersion microscope, eliminating the possibility of vitamin

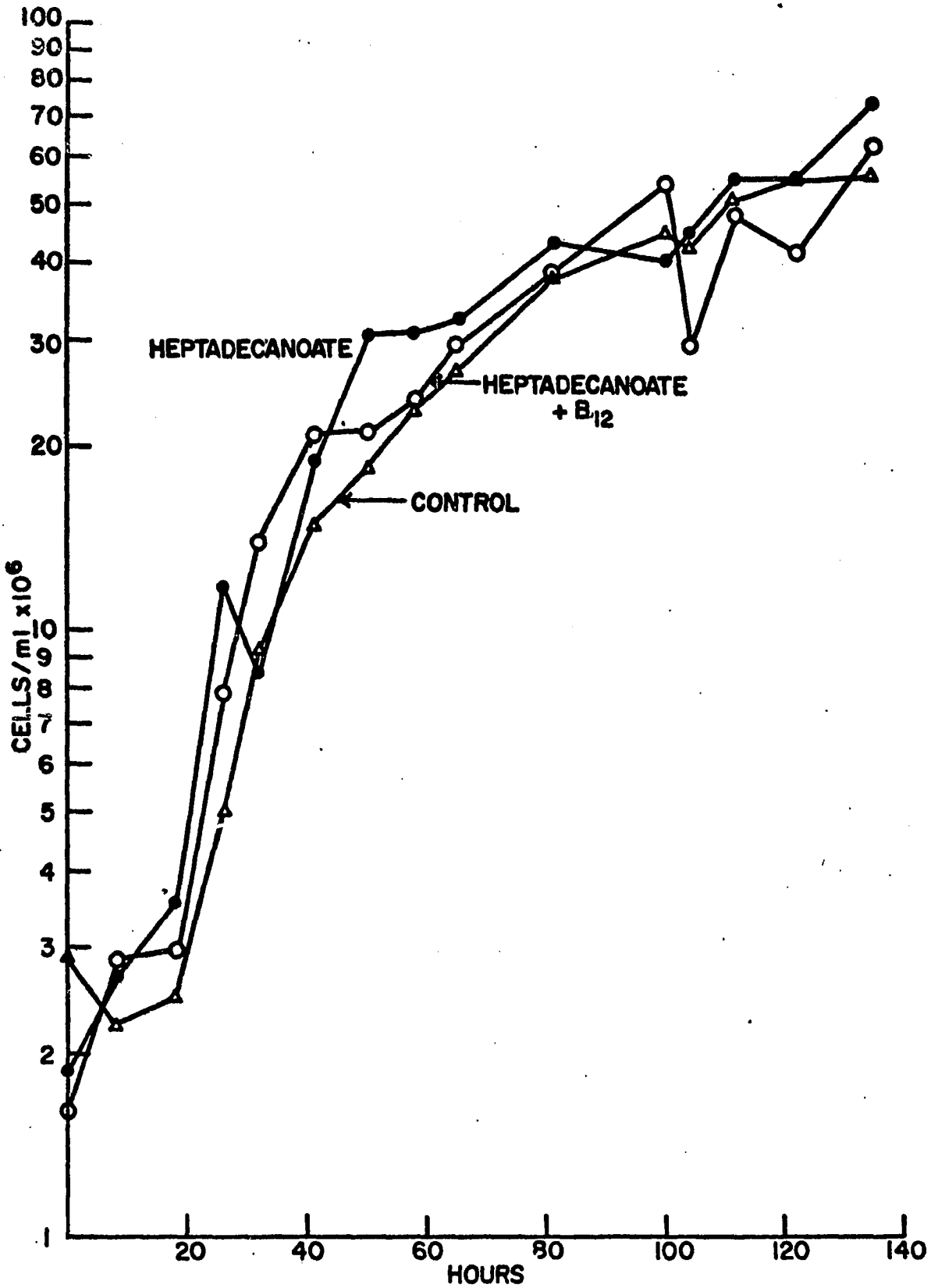
Figure 3. Growth of Ochromonas danica with propionate and propionate plus vitamin B_{12}


100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
CELLS/ml x10⁶
PROPIONATE + B12
CONTROL
PROPIONATE
0
20
40
60
80
100
HOURS

B_{12} synthesis by contaminating bacteria.

The effects of vitamin B_{12} and other acids which form propionate by β-oxidation were also investigated. Valeric acid produced a greater inhibition of growth of O. danica cells than propionate (Table 9). However, this inhibition was not reversed by vitamin B_{12}. There was no detectable effect of heptadecanoic acid on the growth of the cells (Figure 4). This suggested that perhaps the acetate formed by the oxidation was overcoming the propionate effect. To determine if the effect observed was due to the latter possibility, a mixture of sodium acetate and sodium propionate in the ratio that would normally be produced by the β-oxidation of heptadecanoic acid (7:1) was used as substrate. The effect due to propionate was not overcome by acetate (Table 10). This suggested the following possibility: If the activating enzyme system of O. danica for acetate and long-chain fatty acids is constitutive while that of propionate is induced, the addition of acetate or heptadecanoate would not affect the synthesis of the specific activating enzymes, but the addition of propionate would. Furthermore, if the organism metabolizes propionyl CoA by a constitutive pathway not involving vitamin B_{12} (such as via acrylyl CoA as in higher plants) the metabolism of propionyl CoA and hence, heptadecanoate would not require induced enzyme synthesis, but utilization of propionate would.

Figure 4. Growth of Ochromonas danica with heptadecanoate and heptadecanoate plus vitamin B_{12}

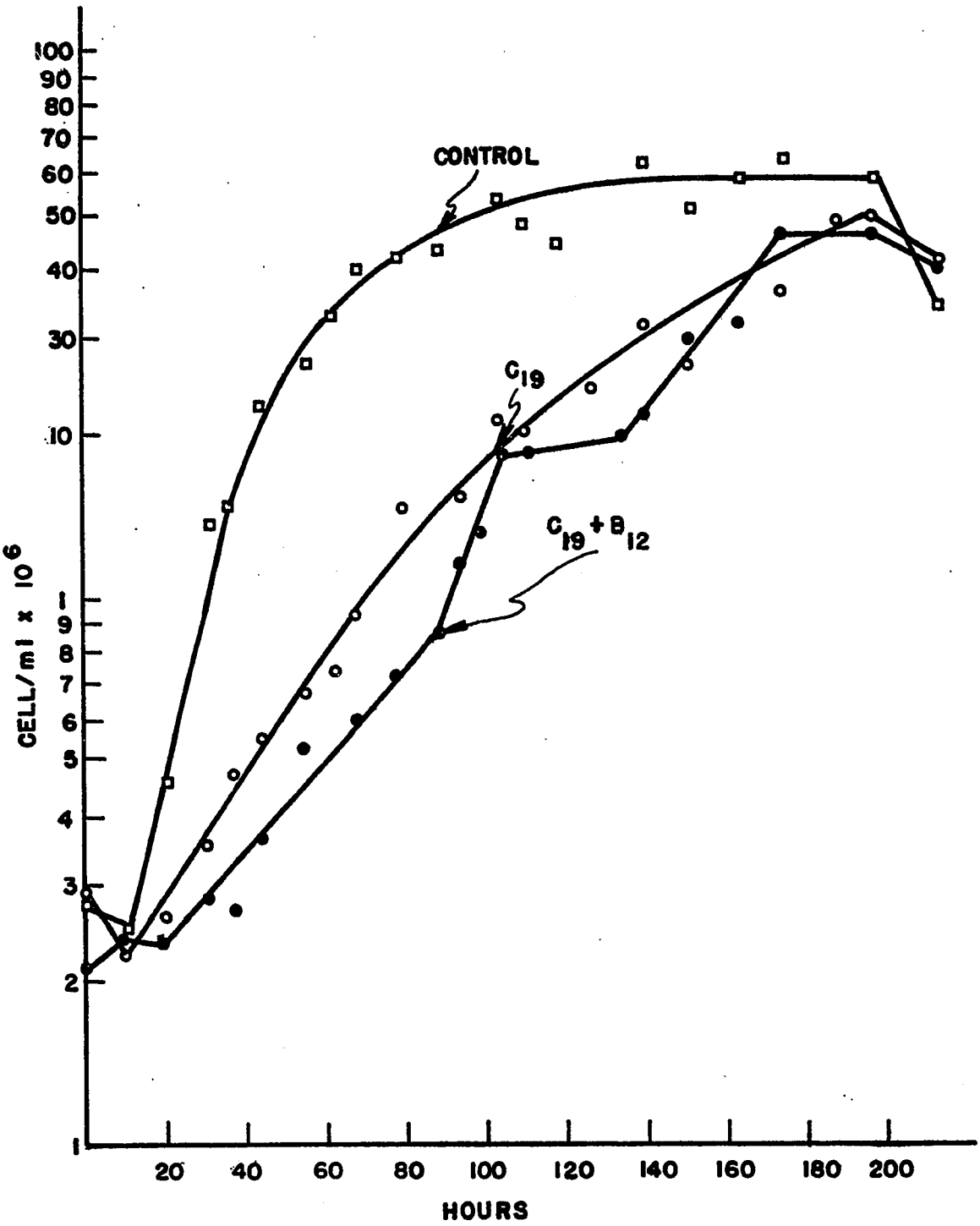

100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
CELLS/ml x10⁶
HEPTADECANOATE
HEPTADECANOATE
+ B12
CONTROL
20
40
60
80
100
120
140
HOURS

Experiments were performed to determine whether O. danica could adapt to propionate. After allowing the cells to grow in a medium containing propionate for more than 100 hours, serial transfers into fresh medium with propionate were made. No adaptation to propionate was shown by the cells (Tables 12a and 12b). An additional observation on the growth of O. danica in the presence of propionate, with or without vitamin B_{12}, was the change in the color of the culture after a period of about one week. The culture grown in propionate with vitamin B_{12} appeared greener than the starting culture while those grown in propionate alone as well as the control cells appeared yellow due to age. This was first thought to be due to a stimulation of growth by vitamin B_{12}. However, the cell counts of the culture grown in propionate with vitamin B_{12} did not differ from the cell count obtained just before the change in color of the culture was observed (i.e., after a period of one week). The vitamin B_{12} was possibly causing an increase in chloroplast synthesis. This hypothesis is supported by the observations of Easley (1969) on the marine algal flagellate, Neochloris pseudo-alveolaris. This organism is similar to O. danica in that it does not require vitamin B_{12} for growth. In a medium supplemented with B_{12} the algae appeared greener. The DNA and RNA content of the cells were shown to have increased. Since DNA is a constituent of the chloroplasts, an enhancement of chloroplast synthesis was suggested.

The utilization of palmitic acid by O. danica was also studied. The growth curve did not differ significantly from that of the control (Table 11). Reazin (1954) has demonstrated that palmitate caused an increase in the oxygen consumption of O. malhamensis cells. The response was, however, faster with cells previously incubated with palmitate, suggesting the formation of adaptive enzymes.

With synthetic cis-9,10-methyleneoctadecanoic acid, an inhibition of growth of O. danica cells was observed (Figure 5). Vitamin B_{12} did not produce a reversal of the inhibition of growth. Since the growth determinations with heptadecanoic acid, which should form propionyl CoA by β-oxidation, did not show inhibition, it is not possible to determine from the growth curves whether or not propionate is the oxidation product. Therefore it is not certain whether inhibition with the cyclopropane fatty acid tested is due to the formation of propionate. There is a possibility that the inhibition could be due to the presence of a DL mixture in the substrate used. Although it has not yet been demonstrated which isomer is the natural lactobacillic acid, the observation of Hofmann, et al. (1957) suggested that it is either the D- or the L- isomer and not a mixture of the two. Synthetic DL-cis-11,12-methylene octadecanoic acid exhibits one half the stimulating activity of natural lactobacillic acid (Hofmann and Panos, 1954) in the presence of suboptimal amounts of biotin. The position of the

Figure 5. Growth of Ochromonas danica in the presence of cis-9,10-methylene octadenanoic acid with and without vitamin B_{12}


CONTROL
C_{19}
$C_{19} + B_{12}$
CELL/ml x 10^6
HOURS
100
90
80
70
60
50
40
30
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
140
160
180
200

cyclopropane ring has little effect on the activity of the cyclopropane acid with L. delbrueckii. The effect of the position of the cyclopropane ring on the activity of this acid in O. danica was not determined.

Therefore, the results of the growth studies on O. danica have shown that although vitamin B_{12} increased the growth of O. danica cells in propionate, it did not produce a similar effect on the growth of the cells in the presence of the other substrates tested which presumably form propionate by β-oxidation. This suggested that either B_{12} does not affect propionyl CoA metabolism as it does propionate metabolism, or that the effect of B_{12} on propionate oxidation is not related to the stimulation of growth of O. danica as was demonstrated with O. malhamensis (Arnstein and White, 1962). The effect of vitamin B_{12} on propionate oxidation with O. danica was not determined in the present study. There is evidence that vitamin B_{12} influences the transmethylation of the methyl group of methionine, thus affecting protein synthesis (Easley, 1969).

Intermediates of Fatty Acid Metabolism

An attempt to detect intermediates of fatty acid metabolism in O. danica was carried out. The constituent fatty acids of the "bound lipids" obtained after extraction of the "free lipids" and alkaline hydrolysis of the cell residue were isolated and their butyl esters identified by gas-liquid chromato-

graphy and mass spectrometry.

Gas-liquid chromatographic analysis of the fatty acids obtained from cells grown in normal growth medium without added substrates showed the presence of propionate and butyrate as well as some unidentified compounds (Figure 6). The column temperature was isothermal at 75°C. At a column temperature of 200°C, longer-chain fatty acids were detected: laurate, myristate, palmitate, stearate and oleate (Figure 7). The large peak appearing before oleate could be linoleate. Hydrogenation experiment was however, unsuccessful. Other unidentified components with retention times between those of propionate and myristate appeared upon comparison with the butyl esters of the fatty acids from butter (Figure 6). These intermediates are probably carbonyl and hydroxy compounds as indicated by the behavior of the butyl esters on thin-layer chromatography. A considerable amount of sample was retained at or near the origin in both of the solvent systems used (benzene-HAc, 99.5: 0.5, Sabo, 1966 and hexane-ethyl ether-HAc-methanol, 90:20:2:3, Brown and Johnston, 1962).

By mass spectrometric analysis of the butyl esters of the O. danica lipids the following constituents were identified: propionate, butyrate, decenoate, myristate, palmitate, oleate, stearate and a compound whose mass spectrum (Figure 8) can be interpreted as that of the butyl ester of β-keto caproate. The prominent peaks characteristic of a ketone and which agree

Figure 6. Gas chromatogram of the butyl esters of the short-chain acids from Ochromonas danica

Column temperature - 75°C

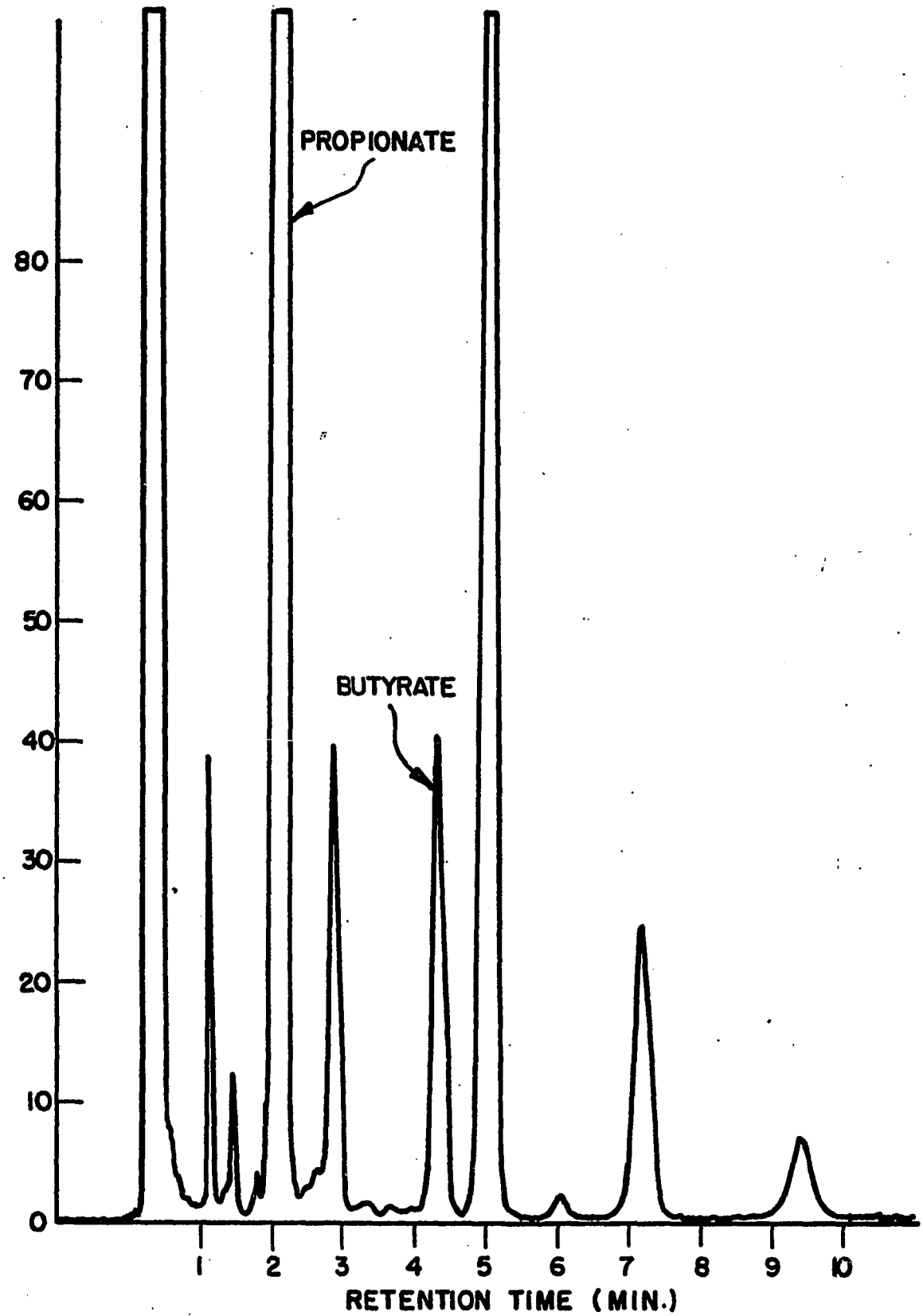

PROPIONATE
BUTYRATE
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
RETENTION TIME (MIN.)

Figure 7. Gas chromatogram of the butyl esters of the long-chain fatty acids from Ochromonas danica

Column temperature - 200°C

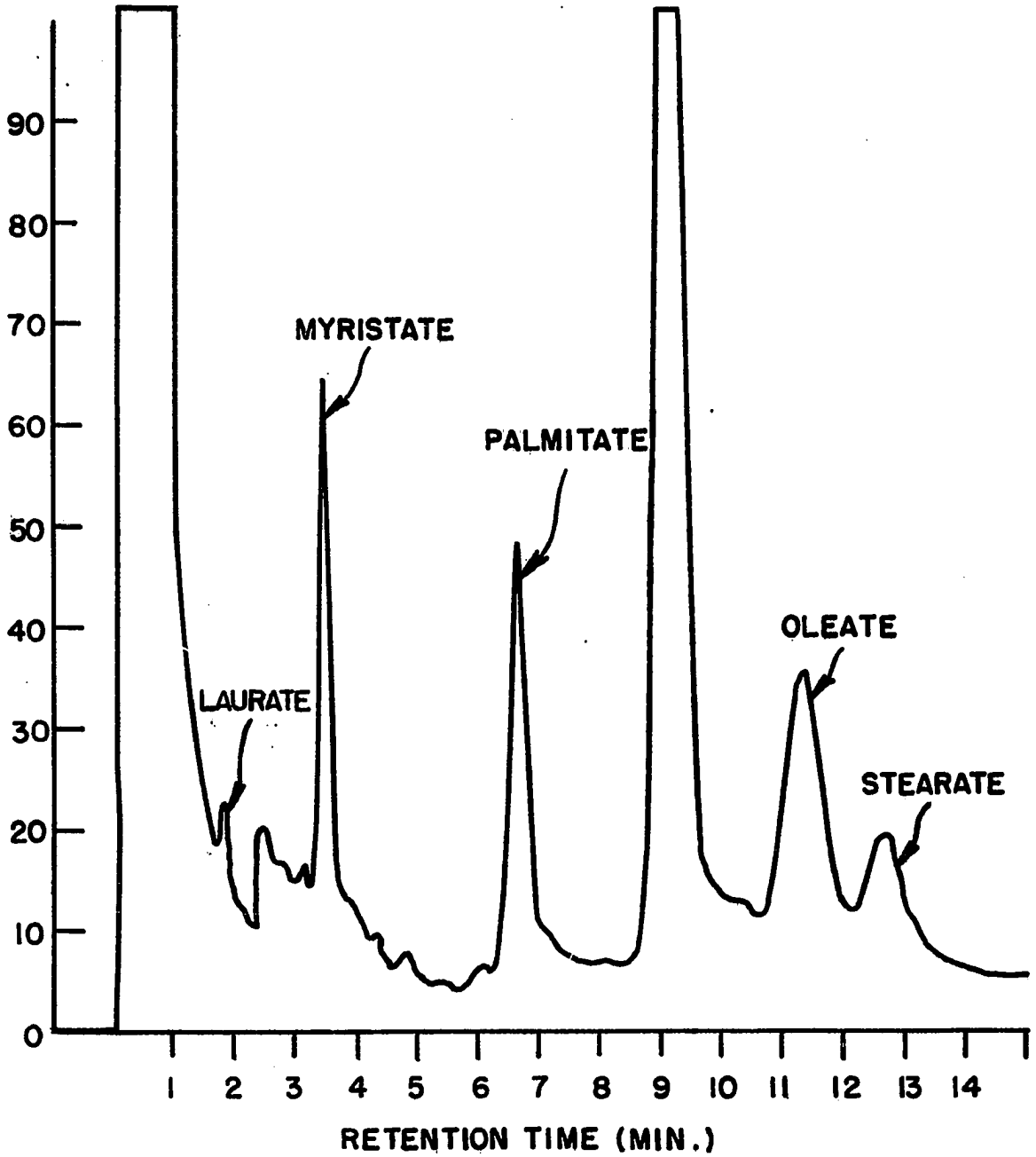
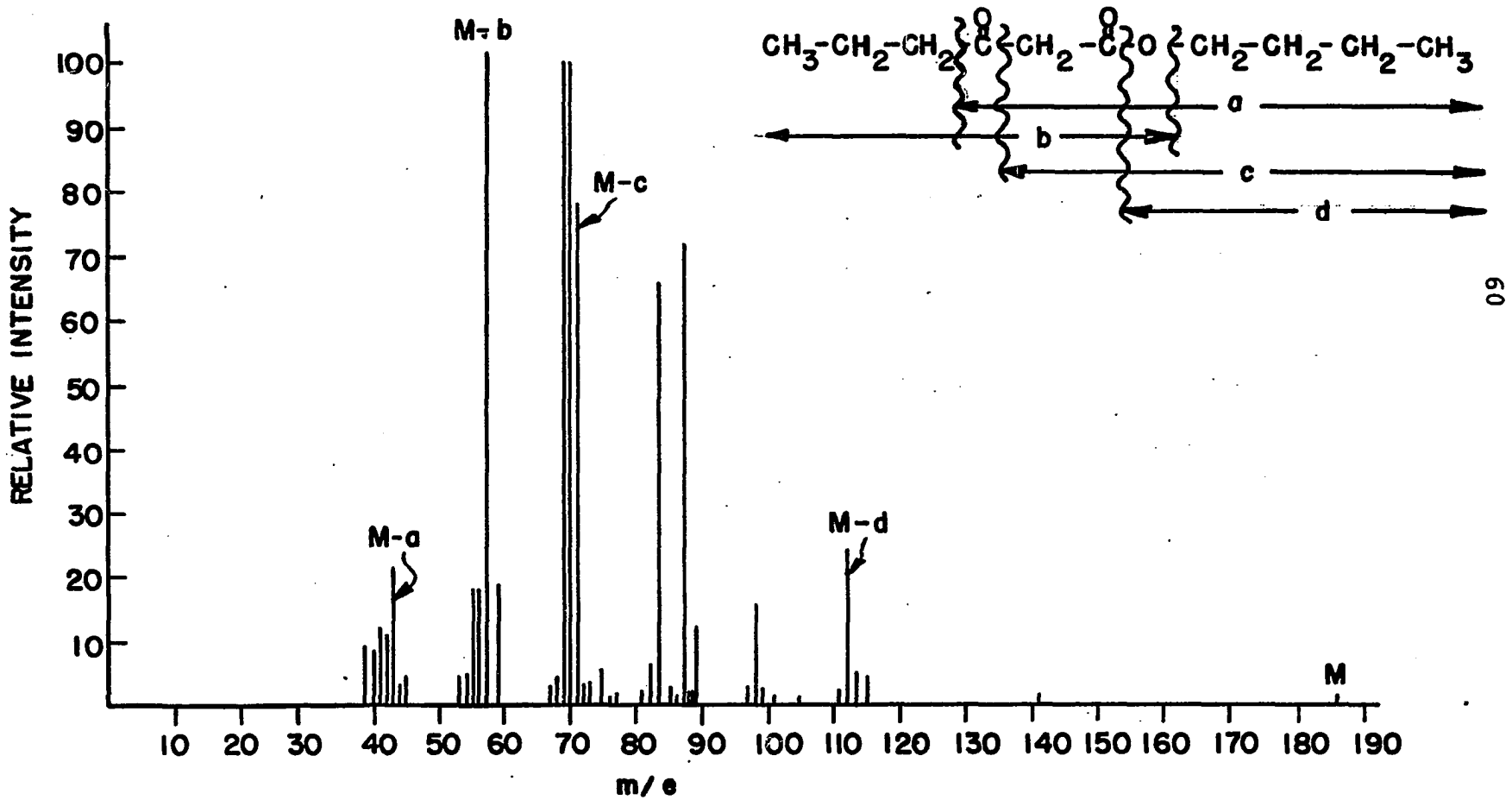

90
80
70
60
50
40
30
20
10
0
MYRISTATE
PALMITATE
OLEATE
LAURATE
STEARATE
1 2 3 4 5 6 7 8 9 10 11 12 13 14
RETENTION TIME (MIN.)

Figure 8. Mass spectrum of a constituent of O. danica "bound lipids" interpreted as that of β-keto caproate


M-b
M-c
M-a
M-d
M
RELATIVE INTENSITY
m/e
a
b
c
d

with those described by Silverstein and Bassler (1967) and Budziekiewicz *et al.* (1967 are the following: a) M-a and M-c (m/e = 43 and 71, respectively), due to the cleavage of C-C bond adjacent to the oxygen atom. b) M-d, due to the loss of butanol ($C_4H_{10}O^+$). M-b (m/e = 57) arises from the cleavage of C-O bond in the ester group. The parent peak is shown at m/e = 186 (M).

When the broken cells suspended in mineral medium were incubated with heptadecanoic acid for one hour and the lipids were analyzed, as described above, no additional intermediates were detected.

Propionate in *O. danica* Lipids

The presence of propionate in the "bound lipids" isolated from *O. danica* was confirmed by gas-liquid and thin-layer chromatography as well as by mass spectrometry.

The chromatogram of the butyl esters from *O. danica* lipids was compared with that of the butyl esters of the fatty acids from butter. A plot was made of the log retention times against the fatty acid chain lengths in the butter ester standard (Figure 9). The retention time of propionate as determined from the graph is 1.9 minutes, coinciding with one of the constituents of *O. danica* lipids (Figure 6). Butyl propionate prepared from sodium propionate gave the same retention time.

By mass spectrometric analysis of the butyl esters of *O. danica* lipids the presence of propionate was shown (Figure 10).

Figure 9. Graph of the log retention times of fatty acids with varying chain lengths from butter

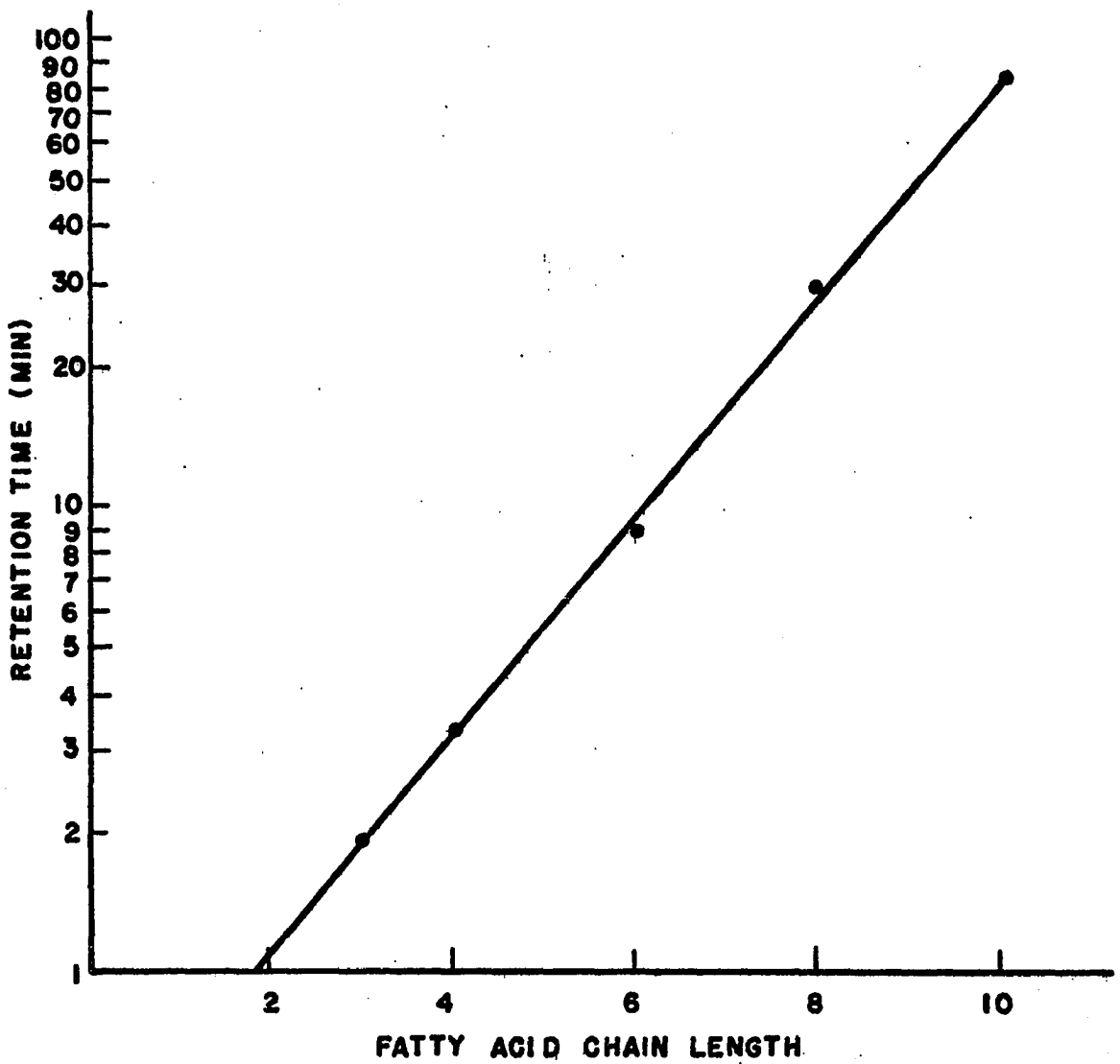
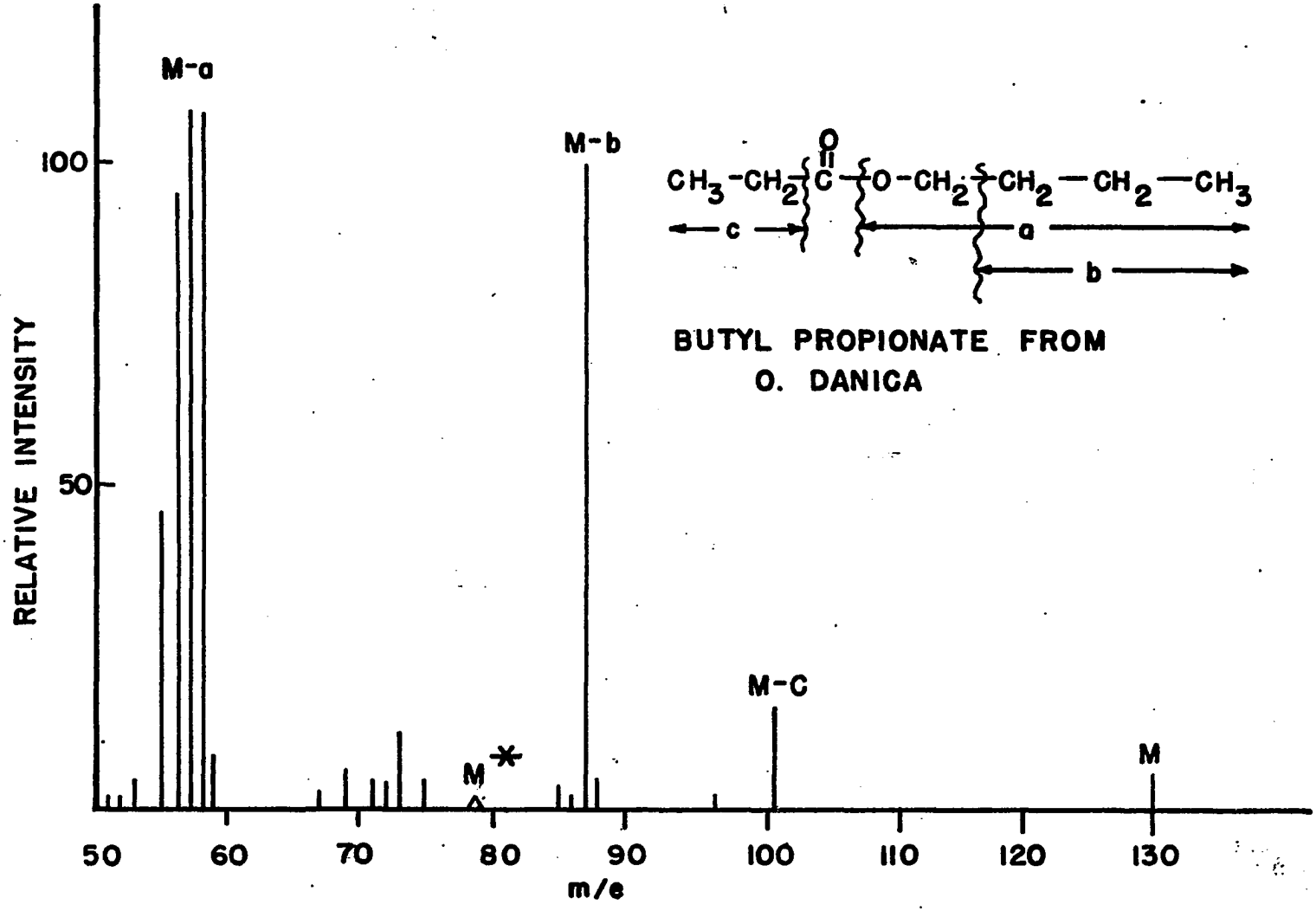

RETENTION TIME (MIN)
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
FATTY ACID CHAIN LENGTH

Figure 10. Mass spectrum of butyl propionate from Ochromonas danica



BUTYL PROPIONATE FROM
O. DANICA

The spectrum agrees with that described for propionate by Budziekiewicz et al. (1967) and Sonneveld (1965). The characteristic peaks for butyl propionate are the following: 1) m/e = 57 (M-a), for the ion $CH_3CH_2C{\equiv}O^+$, a loss of butanol from the ester function. 2) m/e = 78.5 (M^*), a metastable peak corresponding to the loss of 29 mass units from the parent ion with m/e = 130. 3) m/e = 101 (M-c), corresponding to $\overset{O}{\overset{\|}{C}}-O-(CH_2)_3-CH_3$. 4) The molecular ion peak at m/e = 130.

The butyl esters from O. danica lipids were converted into hydroxamates and analyzed by thin-layer chromatography in 3 solvent systems (Figure 11): 1. water-saturated ether: methanol, 9:1; 2. toluene:methanol, 8:2; 3. water-saturated butanol. The standards used were acetohydroxamate (A) and propionylhydroxamate (P). In each solvent a spot coinciding in Rf with propionylhydroxamate was found.

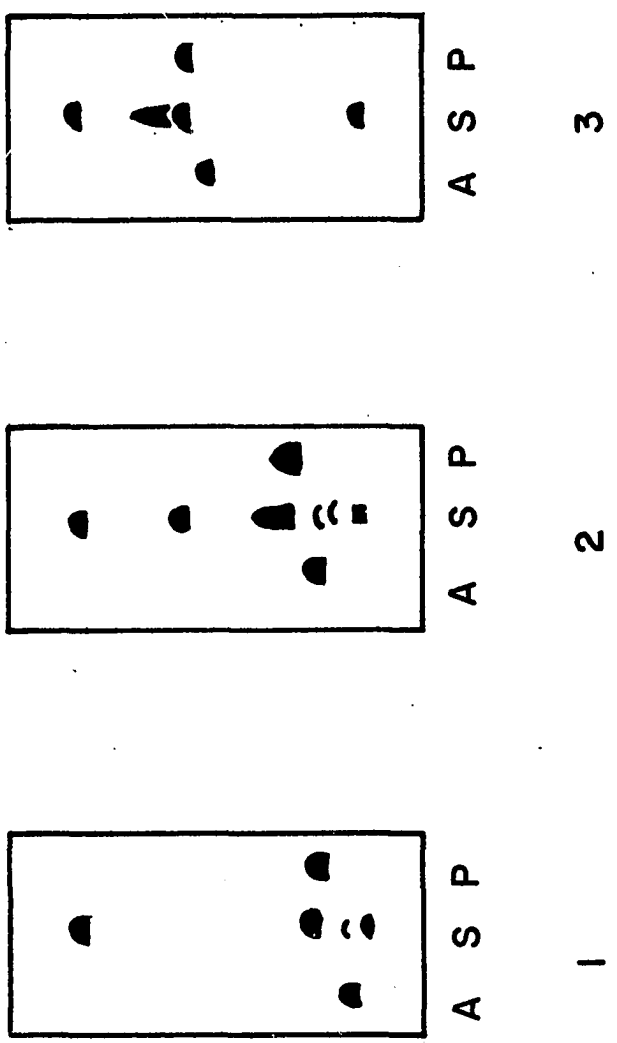
Role of Propionate in O. danica

The significance of the presence of "bound" propionate in O. danica is not known at the present time. The possibility of its incorporation into fatty acids has not been investigated in the present study. However, analysis of the constituent fatty acids of O. danica by Haines et al. (1962) has shown no odd-carbon fatty acids to be present. This indicated that propionate is probably not involved in the initiation of fatty acid synthesis. An attempt was made to detect a propionate

Figure 11. Thin-layer chromatographic analysis of fatty acid hydroxamates from *O. danica*

(1) Water-saturated ether:methanol, 9:1
(2) Toluene:methanol, 8:2
(3) Water-saturated butanol

A- acetohydroxamate standard
S- sample
P- propionylhydroxamate standard


A S P
3
A S P
2
A S P
1

activating enzyme in O. danica which would catalyze the conversion of propionate into propionyl CoA. When O. danica cells grown in normal growth medium with either acetate or propionate were assayed, no detectable difference in enzyme activity was shown with the two substrates. Similar results were obtained with both the supernatant and pellet after centrifugation of the broken cells at 35,000 X g for one hour. The presence of high endogenous substrate levels was indicated by the high readings of the controls without added substrates. Passing the cell supernatant through a Sephadex G-25 column did not improve the results. Elovson and Vagelos (1969) have recently isolated and characterized a mixture of chlorosulfolipids from O. danica which inhibited fatty acid synthesis, particularly the incorporation of malonyl CoA into long-chain fatty acids. This sulfolipid fraction comprises about 15% of the lipids of O. danica cells and apparently acts as a non-specific inhibitor by virtue of its detergent properties. The presence of this inhibitory material in O. danica could explain the failure to detect activating enzyme activity in the present study.

Biosynthesis of Labeled Cyclopropane Fatty Acids

To determine whether cyclopropane fatty acids are metabolized by O. danica, isotope tracer techniques were used. Biosynthesis of cyclopropane fatty acids labeled in the methylene carbon of the ring was carried out. E. coli was chosen as the

source of cyclopropane fatty acids since under appropriate conditions about 20% of the total fatty acid content of the organism consists of cyclopropane acids and since it can be grown easily on a simple defined mineral medium.

E. coli was incubated with L-methionine methyl-^{14}C to obtain cyclopropane fatty acids labeled at the methylene carbon of the ring. Studies with methionine-requiring strains of E. coli grown in the presence of ^{14}C-methyl methionine have shown that there was a preferential incorporation of the methyl carbon of methionine into cyclopropane fatty acids (O'Leary, 1959). This methyl carbon of methionine was later shown to be located exclusively in the ring of the cyclopropane fatty acids (Liu and Hofmann, 1962).

With 20 μC of L-methionine methyl-^{14}C, the total fatty acids isolated from E. coli contained 6.486×10^5 cpm while the unsaponifiable fraction has 5.446×10^5 cpm of radioactivity. The cyclopropane fatty acids were separated by gas-liquid chromatography. The yield of cyclopropane fatty acids was as follows:

C_{17} --- 7.17 mg. 1.032×10^4 cpm

C_{19} --- 6.91 mg. 7.94×10^3 cpm

The cyclopropane fatty acids obtained were identified further by mass spectrometry. The mass spectra of the methyl esters of methylene hexadecanoic acid and methylene octadecanoic acid are shown in Figures 12 and 13. The characteristic ion

Figure 12. Mass spectrum of methyl-cis-9,10-methylene hexadecanoate from *E. coli*

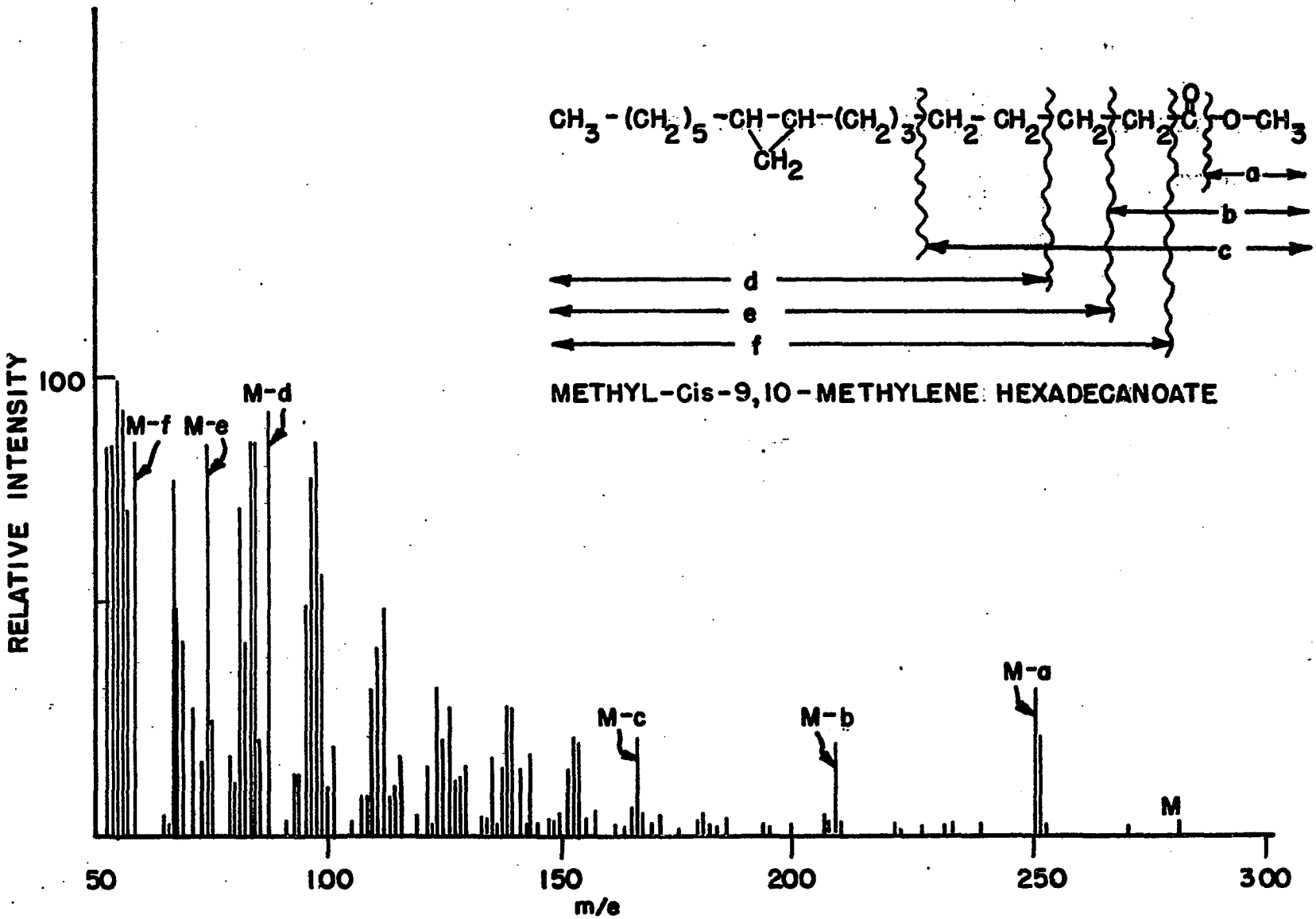
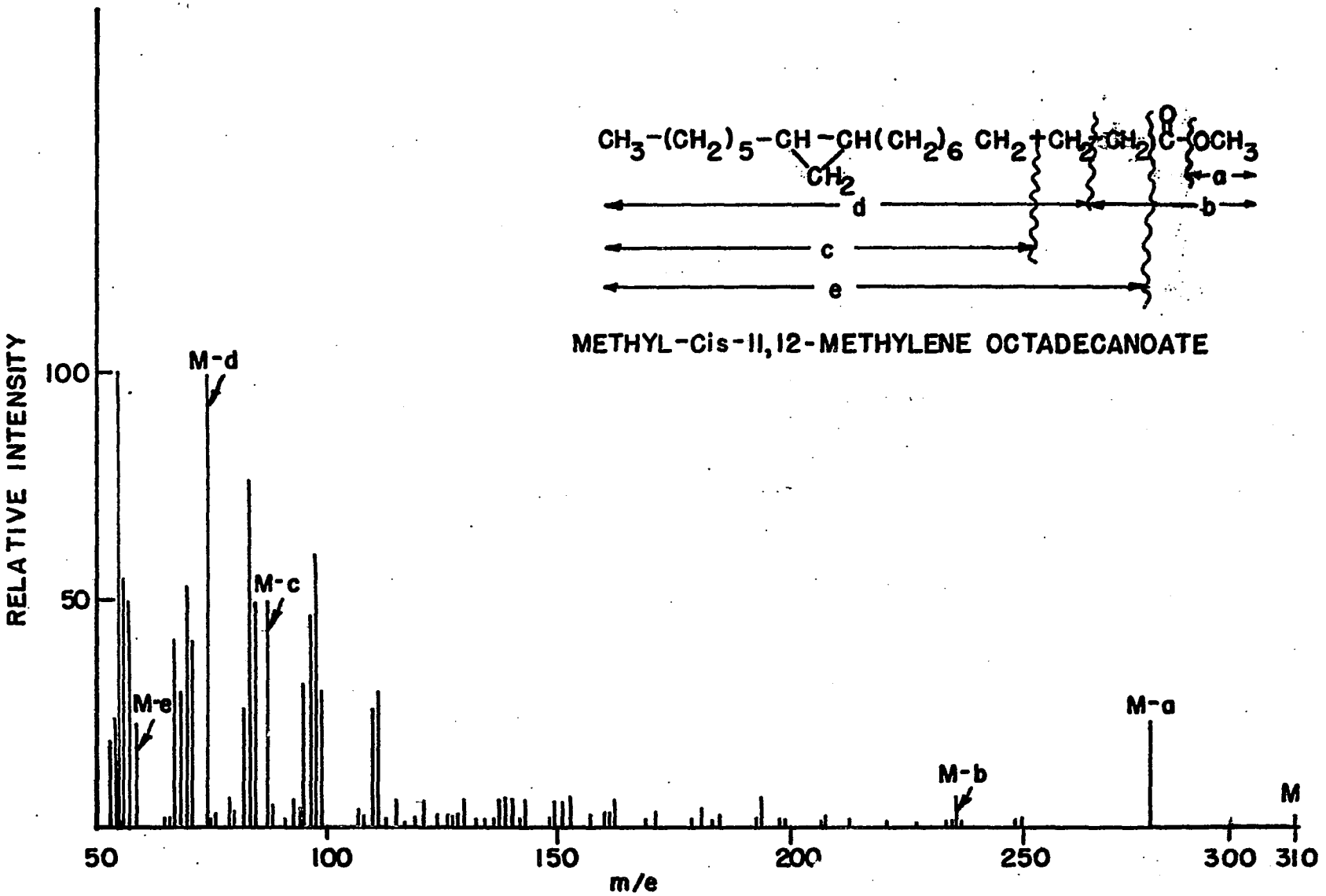

CH3-(CH2)5-CH-CH-(CH2)3-CH2-CH2-CH2-CH2-C-O-CH3
CH2
O
a
b
c
d
e
f
METHYL-Cis-9,10-METHYLENE HEXADECANOATE
RELATIVE INTENSITY
100
M-f
M-e
M-d
M-c
M-b
M-a
M
50
100
150
200
250
300
m/e

Figure 13. Mass spectrum of methyl-cis-11,12-methylene octadecanoate from *E. coli*


CH3-(CH2)5-CH-CH(CH2)6 CH2 CH2 CH2 C OCH3
CH2
O
a
b
d
c
e
METHYL-Cis-11,12-METHYLENE OCTADECANOATE
RELATIVE INTENSITY
100
50
M-d
M-c
M-e
M-b
M-a
M
50
100
150
200
250
300
310
m/e

peaks described by Christie and Holman (1966) and Wood and Reiser (1965) are present: 1) molecule ion peaks at m/e = 282 and 310 (M), respectively for C_{17} and C_{19}. 2) m/e = M-32, (M-a) corresponding to the loss of methanol group from the ester function of the molecule. 3) m/e = M-74 (M-b), due to the removal of the ion $CH_2=\overset{+OH}{\overset{|}{C}}-OCH_3$ formed by hydrogen rearrangement and cleavage of the carbon-carbon bond between the first and second carbon removed from the carbonyl group. 4) M-116, (M-c in Figure 12), corresponding to the loss of the ester group and four carbons from the molecule, 5) the peaks characteristic of the methyl ester function: m/e = 87, due to the ion $CH_2CH_2-COOCH_3^+$; m/e = 74, for the ion $CH_2 = \overset{+OH}{\overset{|}{C}}-OCH_3$; m/e = 59 for $\overset{O}{\overset{\|}{C}}-OCH_3^+$.

Oxidation of Acetate and Fatty Acids by O. danica

The oxidation of sodium acetate 1-^{14}C and fatty acids such as stearic acid-1^{14}C, oleic acid- 1-^{14}C, cis-9,10-methylenehexadecanoate and cis-11,12-methyl methyleneoctadecanoate by O. danica was determined by the measurement of the amount of $^{14}CO_2$ released upon incubation of O. danica cells with the labeled substrates.

With sodium acetate-1^{14}C, a two-hour incubation of about 400 X 10^6 cells in normal growth medium resulted in the release of 1.54 X 10^5 cpm of $^{14}CO_2$, corresponding to the oxidation of 65.4 µmoles of NaAc-1^{14}C (Table 4). In the control, in which

Table 4. Oxidation of acetate and fatty acids by O. danica

Substrate	Radioactivity (cpm)	Incubation period (hours)	^{14}C recovery (cpm)	
			$^{14}CO_2$ released	^{14}C in incubation mixture
NaAc-1-^{14}C	4.4×10^6	2	$1.54 \times 10^5 \pm 39.4$[a]	$9.66 \times 10^5 \pm 22.6$
" (control)	"	2	$1.03 \times 10^4 \pm 42.8$	$4.49 \times 10^6 \pm 28.7$
Oleic acid-1-^{14}C	3080	24	$321.5 \pm .67$	$927.2 \pm .84$
" (control)	"	24	166.3 ± 1.83	$3022.9 \pm .37$
"	"	24	$319.3 \pm .85$	1429.6 ± 1.62
"	"	48	$697.5 \pm .65$	$1056.5 \pm .29$
" (control)	"	48	$172.8 \pm .29$	$2101.5 \pm .55$
"	4880.9	48	712.5 ± 1.16	3323.6 ± 3.09
" (control)	"	48	295.7 ± 0	3926.2 ± 0

[a]Represents the standard deviation in the mean of the radioactivity measurements.

Table 4 (Continued)

Substrate	Radioactivity (cpm)	Incubation period (hours)	^{14}C recovery (cpm)	
			$^{14}CO_2$ released	^{14}C in incubation mixture
Methyl oleate-2-^{14}C	1901.5	24	302.0 ± .71	1938.0 ± .29
"	"	48	342.0 ± .57	1541.9 ± .44
" (control)	"	48	217.1 ± .29	1967.5 ± 0
Methyl C_{17} Δ FA	1060.8	24	263.9 ± .18	232.4 ± 5.3
"	677.3	48	292.6 ± 2.6	155.5 ± .37
" (control)	"	48	114.9 ± 0	265.6 ± .29
Methyl C_{19} Δ FA	964.6	24	286.6 ± .29	256.2 ± 13.8
Methyl C_{19} Δ FA	776	48	379.9 ± 0	176.1 ± .85
" (control)	"	48	255.6 ± .29	360.7 ± .58

the incubation was terminated immediately after the addition of the substrate, only 1.03×10^4 cpm of $^{14}CO_2$ was released, with an oxidation of 4.37 μmoles of the substrate.

When experiments similar to those utilizing labeled NaAc were performed with higher fatty acids such as stearic and oleic acids-1-^{14}C as substrates, very little $^{14}CO_2$ was produced. Moreover, there was no significant difference between the data obtained with the sample and the control (Table 5, below). This indicated that the substrates were only very slightly metabolized under the experimental conditions used. By increasing the length of incubation to about 20 hours, about 10 times more $^{14}CO_2$ was released with stearic acid-1-^{14}C (Table 5). However, the values obtained for the control were still higher than expected.

Table 5. Oxidation of stearic acid by O. danica

Incubation period (hours)	^{14}C recovery (cpm)			
	Sample		Control	
	KOH solution	Incubation mixture	KOH solution	Incubation mixture
2	5.95×10^4	8.96×10^6	3.71×10^4	10.92×10^6
21	6.11×10^5	4.47×10^5	5.24×10^5	3.82×10^5

When the O. danica cells were starved for 2 days in the dark (Sabo, 1966) prior to incubation with 1300 cpm of oleic acid-1-^{14}C, there appeared to be almost complete oxidation of the fatty acid after 48 hours (Table 6, below). The amount of $^{14}CO_2$ released was determined to be 1061 cpm (total solution) when the radioactivity counts were done on a 0.01 ml. aliquot of the KOH solution with a Nuclear-Chicago gas flow counter. However, when a larger aliquot (0.1 ml.) was taken and counted with the Ansitron II scintillation counter only 705 cpm of $^{14}CO_2$ on the average, was produced from about 3000 to 5000 cpm of oleic acid-1-^{14}C (Tables 4 and 13a). The difference is probably due to the error involved in counting which, in the first case was multiplied in magnitude when the total cpm in the total volume of KOH solution was computed from the cpm obtained with the 0.01 ml. aliquot.

Table 6. Oxidation of oleic acid by O. danica

Incubation period (hours)	$^{14}CO_2$ recovery (cpm)	
	Sample	Control
24	564	187
48	1061	187

The amount of methyl oleate oxidized within 24 hours was about equal to the amount of oleic acid oxidized within the same period of time (Tables 4 and 14a). However, unlike oleic

acid, no further significant increase in the amount of oxidation occurred when the incubation was increased to 48 hours.

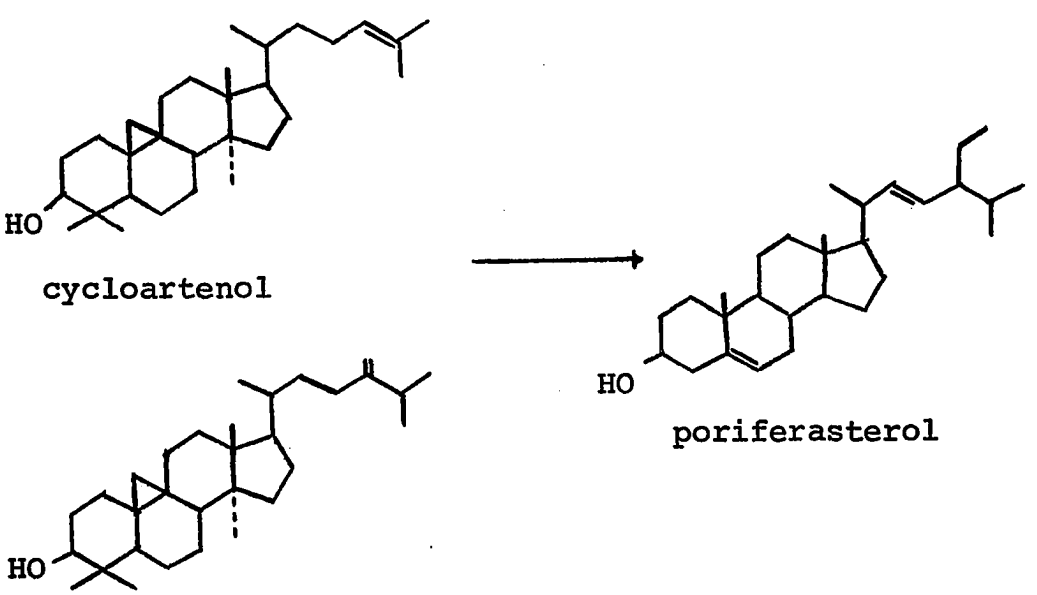
The slow utilization of the long-chain fatty acids like oleic and stearic acids as compared to acetate, which required only an hour or two to show considerable oxidation, could be due to the necessity for the induction of enzymes responsible for the metabolism of these acids. The cells may require a certain period of time for adaptation to the new medium or carbon source. Reazin (1954) has shown by oxygen consumption measurements that O. malhamensis cells which have been adapted to palmitate gave a greater response to palmitate than the non-adapted cells, which had a lag period of 15 minutes. Permeability factors were eliminated when the rate of adaptation was found to be independent of pH. Furthermore, utilization of palmitate was inhibited by irradiation of the cells with U.V. light before the lag period. These findings suggested that induced biosynthesis of enzymes occurred. Polytoma obtusum, a type of flagellate protozoan, has been reported to adapt to butyrate (Cirillo, 1965). When these cells were grown in butyrate, increased thiokinase activity was detected. Cullimare (1966) also described an adaptive butyrate enzyme in Proteteca zopfii, an alga which is able to utilize most monosaccharides and almost all fatty acids. This alga metabolizes butyrate after a lag period of about 3 days. Furthermore, respirometry experiments conducted by Kramer and Hutchens (1969) on

Chilomonas paramecium, a phytoflagellate protozoan similar to O. danica, have shown that when the cells previously grown in acetate were exposed to butyrate, the oxygen consumption was not distinguishable from the endogenous rate within a period of 6 hours. An assay of the fatty acid activating enzyme has demonstrated that the activating enzyme begins to appear at 3 hours with butyrate as substrate (Kramer and Hutchens, 1969). The cells which have been adapted to butyrate also showed higher activities to other fatty acids like hexanoate and octanoate.

With the methyl esters of cyclopropane fatty acids, C_{17} and C_{19}, labeled on the methylene carbon of the ring, as substrates, a smaller percentage of counts of $^{14}CO_2$ was produced as compared with oleic acid and methyl oleate (Tables 4 and 15a). As with methyl oleate as substrate, lengthening the incubation period from 24 to 48 hours resulted in only a slight increase in the amount of cyclopropane fatty acid esters oxidized. With oleic acid the amount of $^{14}CO_2$ absorbed by the KOH solution increased in proportion to the incubation time (Tables 4 and 13a). The difference in the extent of oxidation of C_{17} and C_{19} with a 24 hour period was not significantly large. However, the difference was greater after 48 hours incubation (Tables 4 and 15a).

The results have shown that O. danica is able to oxidize the cyclopropane fatty acids, C_{17} and C_{19}, labeled on the

methylene carbon of the ring, thereby indicating that this organism is capable of cleaving the cyclopropane ring. Although other investigators (Chung, 1966; Wood and Reiser, 1965) have shown that in rats, β-oxidation did not proceed past the cyclopropane ring, no accumulation of cyclopropane fatty acids has been found to occur in microorganisms naturally producing these acids. O. malhamensis, a closely related species, has been shown to contain an enzyme system that can cleave the 9-β-19-cyclopropane ring of some phytosterol precursors such as cycloartenol and 24-methylene cycloartanol to form poriferasterol (Hall et al., 1969).



cycloartenol

poriferasterol

24-methylene cycloartanol

Cycloartenone was also converted into poriferasterol, although the percentage was about 1/3 that of cycloartenol. In support of the above findings is the work of Gershengorn et al. (1968) who have shown small amounts of cycloartenol and 24-methylene cycloartanol as well as the absence of lanosterol in O. malhamensis. Furthermore, enzyme preparations from O. malhamensis were also shown to produce cyclization of squalene 2,3-oxide to cycloartenol (H. H. Rees, unpublished work, cf. Rees et al., 1968). The exact mechanism of the cleavage of the cyclopropane ring of the phytosterol precursors by the enzyme system of O. malhamensis is not clear at the present time.

O. danica probably possesses an enzyme system that can open the cyclopropane rings of cyclopropane fatty acids. The slow utilization of the C_{17} and C_{19} fatty acids (Tables 4 and 15) like oleic acid and methyl oleate indicated that some enzymes might have been formed adaptively or that the necessary enzyme is present in very small amounts. For the methyl esters, slower utilization could be due to the necessity to hydrolyze them first.

The high values of the $^{14}CO_2$ absorbed in the KOH solution of the control flasks were at first thought to be due to the inability of the acid (6N H_2SO_4, used to terminate the incubation), to inactivate the cells. By determining the respiration of the O. danica cells before and after the addition of the acid (Table 7), it was shown that 6N H_2SO_4 was not in all cases

Table 7. Oxygen uptake of O. danica cells in the presence of 6N H_2SO_4 and 10% TCA

Acid	Oxygen uptake (ul.) Time after the addition of the acid (minutes)			
	10	30	60	120
H_2SO_4	0	0	0	0
"	39.5	0	0	0
TCA	0	0	0	0
"	0	0	0	0
--- (control)	6.9	32.1	57	167.1
--- (control)	0	22.1	47.5	150.2

effective in stopping cell respiration. TCA (10%) appeared to be more effective. This acid was used in the experiments with the fatty acids. However, with TCA the control flasks still showed some $^{14}CO_2$ release, although much less than those of the samples (Tables 4, 13a, 14a and 15a). This acid could have reacted in some way with the substrates, producing $^{14}CO_2$. On the other hand, some volatile material aside from CO_2 could have been formed nonenzymatically from the substrates, or may have been present in the substrates to begin with.

The amounts of ^{14}C remaining in the incubation mixture were determined by a scintillation counter using 0.5 ml. aliquots. It is to be noted that the ^{14}C recoveries were less than the starting radioactivities of the substrates considering both data on the $^{14}CO_2$ produced and the ^{14}C remaining in the incubation mixture. Unlike the KOH solution which gave a uniform color in all samples, the incubation mixture showed variable color depending on the substrates. Some corrections were made for the color due to the cells. However, some cells became very light in color after reacting with the substrates and did not match well with the color of the cells used for determining the correction factor. Therefore the values given for the ^{14}C remaining in the incubation mixture (Tables 4, 13b, 14b, and 15b) were not very accurate. The low recovery is probably not due to the incomplete absorbance of the $^{14}CO_2$ by the KOH solution since increasing the volume of the KOH solution did not increase the recoveries of $^{14}CO_2$ from the solutions.

SUMMARY

Attempts were made to demonstrate the metabolism of cyclopropane fatty acids by Ochromonas danica. Cyclopropane fatty acids, cis-9,10-methylene hexadecanoic acid and cis-11, 12-methylene octadecanoic acid, ^{14}C labeled on the methylene carbon of the cyclopropane ring, were prepared biosynthetically using E. coli. It was found that O. danica can convert the methylene carbon of the cyclopropane fatty acids to carbon dioxide. This has not been previously observed in any organism. The extent of oxidation of ^{14}C labeled sodium acetate, stearic, oleic, methyl oleate and the methyl esters of the cyclopropane fatty acids were compared. Sodium acetate-1-^{14}C was extensively oxidized within 2 hours. The fatty acids required longer periods, extending to 48 hours. The extent of oxidation was in this order: sodium acetate > stearic > oleic > methyl oleate > methyl esters of the cyclopropane fatty acids.

Extraction of the lipids from O. danica cells and alkali treatment of the residue released propionate along with some other fatty acids. Propionate was identified by mass spectrometry, gas-liquid and thin-layer chromatography. The significance of propionate in O. danica is not at present known. It may possibly be a constituent of the cell wall, similar to that of some bacteria, with a number of acids attached.

The responses of O. danica to propionate and acetate were investigated. Propionate inhibited the growth of the cells

while acetate had no effect. Vitamin B_{12} reversed the inhibition caused by propionate. Cyclopropane fatty acids caused a small retardation of growth which was not affected by vitamin B_{12}. Valeric acid caused a severe inhibition of growth which was not reversed by vitamin B_{12}, while heptadecanoic acid did not affect the growth. Consequently it was not possible, as originally hoped, to determine from growth curves whether propionate is a product of oxidation of the cyclopropane fatty acids. Attempts to demonstrate propionate activation by broken cell preparations failed, but this may be due to inhibitory surface-active lipids.

In addition to the effect on growth in the presence of propionate, vitamin B_{12} results in greening of old cultures. Probably both observations reflect an effect of the vitamin on protein synthesis.

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APPENDIX

Table 8. Metabolism of propionate by O. danica

Incubation period (hours)	Cell counts per ml. X 10^6		
	Propionate	Propionate + B_{12}	Control
Trial 1			
0	4.45	3.93	3.33
8.8	3.75	4.13	5.00
18.5	7.32	5.35	4.67
24.7	6.30	3.83	4.90
21.9	4.40	4.10	6.95
42.4	4.50	6.60	15.68
48.9	2.10	10.20	19.90
56.2	2.88	21.33	43.05
67.7	2.85	37.10	64.05
72.2	1.93	48.95	50.05
Trial 2			
0	1.40	2.13	1.72
18.5	1.80	2.33	4.05
24.5	1.75	1.93	4.42
41.8	1.88	2.05	13.88
48.0	1.93	3.25	16.98
65.3	1.55	2.75	38.78
72.8	2.00	2.08	39.60
85.5	2.25	2.45	40.95
141.5	2.03	1.70	20.80
Trial 3			
2	2.15	2.40	2.13
11	2.33	3.15	3.90
23	3.40	6.00	6.70
32.5	4.70	8.60	11.50
45.3	11.30	19.80	24.50
52.5	18.20	24.00	33.80
61.0	25.60	35.30	47.0
70	25.60	43.20	39.10
80.5	35.10	43.90	45.80
Trial 4			
0	2.05	1.93	2.20
26.5	2.85	4.95	15.15
75.5	12.50	23.13	36.60
99.0	15.30	41.20	39.60
190.5	18.03	24.48	20.08
238.5	10.50	17.60	21.00

Table 9. Metabolism of valeric acid by O. danica

Incubation period (hours)	(Cell counts per ml. X 10^6)		
	Valeric acid	Valeric acid + B_{12}	Control
2	3.35	2.30	2.13
11	3.83	3.38	3.90
23	3.15	6.70	6.70
32.5	2.18	2.48	11.50
45.3	1.95	3.15	24.50
52.5	1.93	2.70	33.80
61	4.65	3.75	47.00
70	7.20	1.95	39.10
80.5	12.40	2.78	45.80

Table 10. Metabolism of propionate & NaAc + Propionate (7:1) by O. danica

Incubation period (hours)	(Cell counts per ml. X 10^6)		
	Propionate	NaAc + propionate (7:1)	Control
0	2.98	3.15	3.10
8.3	3.43	3.78	3.00
19	2.95	4.38	5.65
27	3.50	5.15	6.90
42	3.65	5.85	21.25
48	1.50	2.48	19.65
56	1.83	3.15	27.80
66.5	3.80	5.10	30.25
74.8	3.45	4.80	46.75
89.8	2.60	2.40	51.35
96.8	4.20	2.83	65.63
103.3	4.50	4.15	63.00
118.8	2.45	3.40	84.53
127.3	3.30	5.40	70.88
145	6.20	5.80	124.43
152	7.05	5.95	121.80
162.5	6.55	6.40	79.80
168.8	3.60	5.10	89.25
185.8	21.45	6.00	81.90
210	34.65	6.0	70.35
234	38.67	23.25	53.55
258	49.35	60.70	46.25

Table 11. Metabolism of palmitic acid by O. danica

Incubation period (hours)	(Cell counts per ml. X 10^6)		
	Palmitic acid	Palmitic acid + B_{12}	Control
0	2.15	2.50	2.45
7	2.35	2.25	2.70
17.7	4.90	7.00	3.10
24	3.60	8.70	2.55
31	12.40	20.80	6.60
42.3	30.60	16.50	11.70
47.4	21.00	18.20	23.10
52.3	35.40	29.20	22.80
66.5	54.90	55.80	44.55
72.0	55.55	61.88	52.80
89.2	64.35	78.10	84.70
96	85.60	93.60	64.80

Table 12a. Growth of Ochronomos danica in propionate (effect of preincubation with propionate) - Trial 1

A. First exposure to propionate

	Incubation period (hours)								
	0	4	22.5	28.5	45.5	69.8	76	93.5	123
	(Cell counts/ml. X 10^6)								
Propionate	2.47	3.08	6.45	12.05	18.20	23.18	30.25	31.85	25.25
Control	2.95	2.70	16.38	21.38	58.30	51.03	68.25	78.20	75.22

B. Second exposure to propionate

	Incubation period (hours)								
	0	9	34	46.5	52.3	69	77	115	141
	(Cell count/ml. X 10^6)								
Propionate	1.20	1.85	9.60	4.73	9.00	14.18	18.15	26.95	25.85
Control	6.45	2.08	3.36	14.75	19.60	32.85	44.20	48.00	51.97

Table 12b. Growth of Ochromonas danica in propionate (effect of preincubation with propionate) - Trial 2

A. First exposure to propionate

	Incubation period (hours)							
	0	22	29.5	48	74.5	117.8	144	168
	(Cell count/ml. X 10^6)							
Propionate	4.20	4.33	8.00	9.50	28.18	46.20	61.60	50.93
Propionate + B_{12}	3.75	6.75	11.50	24.75	43.88	66.50	115.20	87.75
Control	2.48	15.63	15.96	38.50	60.22	97.65	118.30	120.90

B. Second exposure to propionate

	Incubation period (hours)				
	0	20.3	55.2	93.8	119
	(Cell count/ml. X 10^6)				
Propionate	2.05	4.73	19.13	51.20	34.45
Propionate + B_{12}	2.75	7.45	15.93	58.30	52.00
Control	3.83	11.25	44.0	76.70	86.10

Table 13a. Oxidation of oleic acid by O. danica

Sample number	Radio-activity of substrate (cpm)	Incubation period (hours)	$^{14}CO_2$ released: Counts per 0.1 ml. per 4 minutes — Individual counts[a]	Average	Average corrected[b]	Average less background	Total[c] cpm
1	3080	24	656 645 622 602 681 670 645 634	644.9	702.9	642.9	321.5
2 control	3080	24	303 281 321 304 482 370 458 362	360.1	392.5	332.5	166.3
3a	3080	24	694 668 660 641 436 426 401 384	538.8	587.3	525.3	393.99
3b	3080	24	398 388 321 317	356.0	388.0	326.0	244.5
4a	3080	48	689 615 650 574	632.0	688.9	628.9	516.7
4b	3080	48	419 409 428 420 397 396	416.0	453.4	396.4	660.7

4b	3080	48	608 413 319 (per 0.03 ml.)	553 396 314	390.9	426.1	366.1	915.1
5 (control)	3080		279 265	267 262	268.3	292.4	230.4	172.8
6a	4880.9	48	798 736	807 726	766.8	835.8	778.8	584.1
6b	4880.9	48	1171 1142	1148 1124	1081.0	1178.3	1129.3	840.9
7 (control)	4880.9		385 350	363 325	355.8	397.8	340.8	255.6

[a]Counting rates on the dual-channel counter.

[b]Corrected for quenching.

[c]Counts for total volume of KOH solution.

Table 13b. Oxidation of oleic acid by O. danica

Sample number	Radio-activity of substrate (cpm)	Incubation period (hours)	^{14}C in incubation mixture: Counts per 0.5 ml. per 4 minutes — Individual counts[a]	Average	Average corrected[b]	Average less background	Total[c] cpm
1	3080	24	1079 1040 1091 1067 1060 1034	1061.8	1486.5	1426.5	927.2
2 (control)	3080	24	3891 3842 3885 3813 3911 3846	3364.7	4710.6	4650.6	3022.9
3a	3080	24	1853 1824 1716 1734 1905 1879 1803 1783	1815.2	2541.3	2479.3	1363.6
3b	3080	24	2029 1987 1984 1947	1986.5	2781.1	2719.1	1495.5
4a	3080	48	1458 1443 1462 1451 1442 1423	1427.0	1997.8	1937.8	1065.8
4b	3080	48	1408 1476 1366 1361	1402.8	1963.8	1903.8	1047.11

5 (control)	3080		2815 2799 2790 2822	2769 2762 2757 2777	2786.4	3901.0	3821.0	2101.5
6a	4880.9	48	5503 5827 4209[d] 4223	5372 5692 4107 4146	5316.6	7443.2	7386.2	3693.1
6b	4880.9	48	6085 5951	5981 5844	5965.3	8351.4	8294.4	4147.2
7 (control)	4880.9		5724 5714 5744	5614 5607 5593	5666	7932.4	7852.4	3926.2

[a]Counting rates on the dual-channel counter.

[b]Corrected for quenching.

[c]Counts for total volume of incubation mixture.

[d]Counts per o.4 ml. per 4 minutes.

Table 14a. Oxidation of methyl oleate by O. danica

Sample number	Radio-activity of substrate (cpm)	Incubation period (hours)	$^{14}CO_2$ released: Counts per 0.1 ml. per 4 minutes — Individual counts[a]	Average	Average corrected[b]	Average less background	Total[c] cpm
1a	1901.5	24	495 462 427 397 411 377 373 351	405.9	442.4	380.4	285.3
1b	1901.5	24	555 533 503 489 405 387 364 354	448.4	488.8	426.8	320.1
2a	1901.5	48	545 487 436 400	467.0	509.0	449.0	336.8
2b	1901.5	48	416 388 392 373	392.3	427.6	367.6	275.7
2b	1901.5	48	323 299 307 291 310 306 312 294	305.3	332.7	275.7	413.6
3 (control)	1901.5		355 340 306 290	322.5	351.5	289.5	217.1

[a]Counting rates on the dual-channel counter.

[b]Corrected for quenching.

[c]Counts for total volume of KOH solution.

Table 14b. Oxidation of methyl oleate by O. danica

Sample number	Radio-activity of substrate (cpm)	Incubation period (hours)	^{14}C in incubation mixture: Counts per 0.5 ml. per 4 minutes — Individual counts[a]	Average	Average corrected[b]	Average less back-ground	Total[c] cpm
1a	1901.5	24	2730 2708 2654 2640	2683.0	3756.2	3694.2	2031.8
1b	1901.5	24	2462 2442 2435 2418	2439.3	3415.0	3353.0	1844.2
2a	1901.5	48	2792 2771 2771 2743	2769.3	-	2709.3	1490.1
2b	1901.5	48	2954 2994 2917 2965	2957.5	-	2897.5	1593.6
3 (control)	1901.5		2629 2586 2661 2622 2570 2534 2667 2624	2661.5	3726.2	3646.2	2005.5

[a]Counting rates on the dual-channel counter.

[b]Corrected for quenching.

[c]Counts for total volume of incubation mixture.

Table 15a. Oxidation of cyclopropane fatty acids by O. danica

Sample number	Substrate	Radioactivity of substrate (cpm)	Incubation period (hours)	$^{14}CO_2$ released: Counts per 0.1 ml. per 4 minutes — Individual counts[a]	Average	Average corrected[b]	Average less background	Total[c] cpm
1	C_{17}	1060.8	24	614 569 482 466 576 528	539.2	587.7	527.7	263.9
2	C_{19}	964.6	24	628 585 571 539	580.8	633.1	573.1	286.6
3	C_{17}	677.3	48	570 523 416 381 373 356 334 312 437 400	410.2	447.1	390.1	292.6
4 (control)	C_{17}	677.3		250 230 219 200 200 185	214.0	233.3	153.3	114.9
5	C_{19}	776	48	541 510 527 490	517	563.6	506.6	379.9
6 (control)	C_{19}	776		385 363 350 325	355.8	397.8	340.8	255.6

[a]Counting rates on the dual-channel counter.

[b]Corrected for quenching.

[c]Counts for total volume of KOH solution.

Table 15b. Oxidation of cyclopropane fatty acids by O. danica

Sample number	Substrate	Radioactivity of substrate (cpm)	Incubation period (hours)	^{14}C in incubation mixture: Counts per 0.5 ml. per 4 minutes — Individual counts[a]	Average	Average corrected[b]	Average less background	Total[c] cpm
1	C_{17}	1060.8	24	331 321 260 258 316 303	298.2	417.5	357.5	232.4
2	C_{19}	964.6	24	323 309 308 303 399 378	324.4	454.2	394.2	256.2
3	C_{17}	677.3	48	335 317 276 255 251 242	279.3	391.0	311.0	155.5
4 (control)	C_{17}	677.3		704 631 532 486	588.3	641.2	585.2	292.1
5	C_{19}	776	48	449 434 413 399 396 383 406 394	409.3	-	352.3	176.1
6 (control)	C_{19}	776		848 780 775 711	778.5	-	721.5	360.7

[a]Counting rates on the dual-channel counter.

[b]Corrected for quenching.

[c]Counts for total volume of incubation mixture.