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Biosynthesis of Unsaturated Fatty Acids in a Psychrotrophic Bacterium, *Pseudomonas* Sp. strain E-3

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Abbreviations: fatty acids are denoted by shorthand designation: C number of carbons: number of double bond. Position of double bond is indicated by delta (Δ) nomenclature, which describes a bond position relative to the carboxyl carbon. Otherwise unsaturated fatty acid without Δ indicates no identification of double bond position; ACP, acyl carrier protein; NAC, N-acetylcysteamine; TLC, thin layer chromatograhy.

Abstract

Biosynthetic pathway of unsaturated fatty acids in a psychrotrophic bacterium, Pseudomonas sp. strain E-3, was examined. The results obtained by gas-chromatography-mass-spectrometry demonstrated that the main unsaturated fatty acids in this bacterium are $\Delta 9\text{-}C_{16:1}$ and $\Delta 11\text{-}C_{18:1}$.

Radioactive $\Delta 9\text{-}C_{16:1}$ and $\Delta 11\text{-}C_{18:1}$ were aerobically produced, but not anaerobically, from exogenous [1-^{14}C]$C_{12:0}$, [1-^{14}C]$C_{14:0}$, or [1-^{14}C]$C_{16:0}$. In the case of [1-^{14}C]$C_{16:0}$, radioactivity in the product $C_{16:1}$ was exclusively distributed on carboxyl carbon. Under the anaerobic conditions, proportion of $C_{16:1}$ in fatty acids newly synthesized from acetate was very low, whereas those of $C_{16:0}$ and $C_{18:1}$ were higher than that of aerobic conditions. The membrane fraction was found to have the activity to desaturate the [1-^{14}C]$C_{16:0}$-CoA in the presence of NADPH. Furthermore, the membrane fraction could form $\Delta 11\text{-}C_{18:1}$ from $C_{16:1}$-CoA and [^{14}C]acetyl-CoA. These results indicate that $C_{16:0}$ is aerobically desaturated to $\Delta 9\text{-}C_{16:1}$ (aerobic pathway), and the $\Delta 9\text{-}C_{16:1}$ is elongated to $\Delta 11\text{-}C_{18:1}$.

On the other hand, fatty acid synthetase was partially purified, which produced saturated and unsaturated fatty acids (anaerobic pathway). This is first report which demonstrate the co-existence of two pathways for the biosynthesis of unsaturated fatty acids in one organism.

3-Decynoyl-NAC, specific inhibitor of the anaerobic pathway, completely inhibited the synthesis of unsaturated

fatty acid by partially purified fatty acid synthetase. However, the inhibitor did not blocked the unsaturated fatty acid synthesis in whole cells and reconstituted system which contained fatty acid synthetase and the membrane fraction. High concentration of 3-decynoyl-NAC completely inhibited the cell growth, but the growth inhibition was completely restored by addition of $\Delta 9\text{-}C_{18:1}$ and partly recovered by addition of $C_{16:0}$ to the growth medium. Thus, the aerobic pathway can synthesize unsaturated fatty acids to sustain cell growth under the condition in which the anaerobic pathway was blocked.

Pseudomonas sp. strain E-3 grew in the medium which contained a fatty acid or hydrocarbon as the sole carbon source. Fatty acid composition of phospholipids and growth curve were affected by the carbon source. The added fatty acid and its desaturation product were increased in phospholipids. Saturated and unsaturated odd-chain fatty acids appeared in phospholipids of the cells grown on $C_{11:0}$ or $C_{15:0}$, though odd-chain fatty acid was not synthesized de novo. The in vivo desaturase activity was increased when the cells were grown on the carbon source which was the enzyme substrate, whereas the activity was lowered when the carbon source was unsaturated fatty acid. Composition of de novo synthesized fatty acids was altered to save the synthesis of the supplemented one. Therefore, this bacterium utilizes efficiently exogenous long chain fatty acid for the membrane lipids synthesis with and without modification (desaturation and elongation). Fatty acid

metabolism was regulated in accordance with the cellular economy.

Introduction

Temperature-dependent changes in the ratio of unsaturated to saturated fatty acids in membrane lipids have been considered to be one of the major control mechanisms to control membrane fluidity [1, 2]. Adequate membrane fluidity is necessary for the membrane function such as membrane transport, and activities of several membrane-bound enzymes [3, 4]. To compensate the decrease of membrane fluidity, many exothermic organisms increase the proportion of unsaturated to saturated fatty acid in membrane lipids at lower temperature [5-12].

Many bacterial membrane lipids contain saturated and monounsaturated fatty acids with chain length of C_{14} to C_{18} [13-15]. Some species have branched chain fatty acids or cyclopropane fatty acids instead of unsaturated ones [13, 16]. Few bacteria produce polyenoic fatty acids [17, 18].

With the exception of archaebacteria, all organisms synthesize fatty acids by fundamentally the same mechanism. The synthetic reactions consist of two steps catalyzed by two enzyme systems. The first committed step is the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (Fig 1. A) [19]. $C_{16:0}$ can then be synthesized by a series of reactions catalyzed by a fatty acid synthetase from one molecule of acetyl-CoA (primer) and 7 molecules of malonyl-CoA [19]. Initially, acetyl and

malonyl thioesters condense to form the /3-keto thioester with the concerned loss of the free malonyl carboxyl group as CO_2 (Fig. 1 E). Subsequent reduction of /3-keto group to a /3-hydroxy group (Fig. 1 F), dehydration of this to a trans-α,/3-enoyl intermediate (Fig. 1 G), and reduction of the double bond (Fig. 1 H) complete the first cycle to yield butyryl thioester. A second cycle begins when butyryl and malonyl thioesters condense to yield CO_2 and /3-ketohexanoyl thioester. Seven such cycles yield $C_{16:0}$ with the overall stoichiometry of:

$$CH_3CO\text{-}S\text{-}X + 7HOOCCH_2CO\text{-}S\text{-}X + 14NADPH + 14H^+ \longrightarrow$$
$$CH_3(CH_2)_{14}CO\text{-}S\text{-}X + 7CO_2 + 14NADP^+$$

(X is CoA, ACP, or enzyme)

The chemical mechanism of fatty acid synthesis is common in all organisms. However, the molecular architecture of fatty acid synthetase does vary with phylogenically conceivable way and can be broadly separated into two distinct groups, designated type I and type II systems. Type I systems are stable (non dissociable by cell fractionation) multifunctional enzyme complexes. All the activities of fatty acid synthetase are located in one protein. Such stable, multifunctional complexes are found in animals, fungi, protozoa, and in several bacteria [20]. All type I fatty acid synthetase, except that of Brevibacterium ammoniagenes, produce only saturated fatty acids [21].

The distinguishing characteristic of type II fatty acid synthetase is that each chemical reaction involved in fatty

acid synthesis is catalyzed by separate monofunctional polypeptides. Although it is possible that these monofunctional enzymes may associate *in vivo*, such association would be noncovalent and is not necessary for activity. Type II fatty acid synthetase have been found in blue-green algae, higher plants, and various bacteria [20, 22, 23].

For the biosynthesis of unsaturated fatty acids, all organisms so far examined utilize either of two pathways. The first pathway, found in all anaerobic and some aerobic bacteria, produces long chain monounsaturated fatty acids by the elongation of medium chain length *cis*-3-unsaturated intermediates. *Trans*-monounsaturated derivatives are formed temporarily during every steps of fatty acid elongation by C_2-unit (Fig. 1 G), and are usually reduced to form saturated fatty acids. Only at the chain length of C_{10} or C_{12}, the *trans*-intermediate is isomerized to *cis*-3-intermediate, and is then elongated to C_{16} and C_{18} unsaturated fatty acids (Fig. 2). The overall process proceed under aerobic conditions as well as under anaerobic conditions [24, 25]. Thus, it is generally called the anaerobic pathway. In most cases, C_{10}-*cis*-3-unsaturated intermediate is elongated to $\Delta 9$-$C_{16:1}$ and $\Delta 11$-$C_{18:1}$. Exceptionally, C_{12}-*cis*-monounsaturated intermediate is elongated to form $\Delta 9$-$C_{18:1}$ in *Brevibacterium ammoniagenes* [21]. A specific inhibitor of the anaerobic pathway have been known. 3-Decynoyl-NAC irreversibly bind to /3-hydroxydecanoyl-ACP dehydrase, and inhibits anaerobic

formation of unsaturated fatty acids [25, 26]. By interfering the anaerobic pathway, 3-decynoyl-NAC inhibits the growth of E.coli [27]. On the other hand, this substance did not inhibit the production of unsaturated fatty acid by the aerobic pathway [27].

The second pathway, which operates in certain aerobic bacteria, blue-green algae, and all eukaryotes, involves the oxygen-mediated introduction of double bond into a pre-existing long-chain saturated fatty acid. This mechanism, which is also utilized in polyunsaturated fatty acids synthesis, is known as the aerobic pathway or fatty acid desaturation [25, 28, 29]. Conversion of saturated fatty acid to unsaturated one is catalyzed by a distinct enzyme from fatty acid synthetase, fatty acid desaturase.

Based on enzymatic properties, the desaturase systems which is concerned in monounsaturation are categorized into two groups. Fatty acid desaturase of rat liver and yeast are membrane bound, and catalyses the desaturation of $C_{16:0}$- or $C_{18:0}$-CoA [29, 30]. The enzyme system consists of 3 amphipathic protein components; cytochrome b_5 reductase, cytochrome b_5, and the desaturase. Purification and extensive characterization of this enzyme have been carried out [30]. Another type of the desaturase is found in plants. Monounsaturation in higher plants is catalyzed by soluble enzymes that utilize $C_{18:0}$-ACP as the substrate [31]. Its soluble electron transport system includes ferredoxin rather than cytochrome b_5.

Information about polyunsaturation is hardly available

mainly because of difficulty to solubilize the enzyme from membrane. All experiments about polyunsaturation have been performed with membrane fraction as the enzyme source. Desaturation of $C_{18:1}$ and $C_{18:2}$ in spinach proceeds after formation of complex lipid, digalactosylglycerol [31].

Some enzymatic properties of bacterial desaturase systems have been examined. The system from Mycobacterium smegmatis is particulate and utilizes $C_{16:0}$- or $C_{18:0}$-CoA [32]. The $C_{16:0}$-CoA desaturase activity has been also detected in the membrane fraction of Alcaligenes faecalis [33, 34]. Cofactor requirement and involvement of cytochrome b_5 of this enzyme are similar to rat liver desaturase system. In this case, however, $\Delta 11$-$C_{18:1}$ is produced rather than $\Delta 9$-$C_{18:1}$. The $\Delta 11$-$C_{18:1}$ is considered to be elongated from aerobically produced $\Delta 9$-$C_{16:1}$. The need of iron in desaturation reaction was demonstrated from whole cell studies of Bacillus megaterium [35]. The substrate for $\Delta 5$ desaturation in this bacterium appears to be $C_{16:0}$ of phosphatidylglycerol [36]. Desaturation of phospholipid-bound $C_{16:0}$ have been also reported with Micrococcus cryophilus [37].

Sheuerbrandt and Bloch [38] reported that Pseudomonas fluorescens synthesizes unsaturated fatty acids by the anaerobic pathway. Since then, the bacteria belonging to the genus Pseudomonas have been referred to as representatives having the anaerobic mechanism, though they are strictly aerobic bacteria. On the other hand, some workers [16, 39] suggested the possibility of presence of

the aerobic pathway in Pseudomonas species.

The molecular mechanism of temperature regulation by which proportion of unsaturated fatty acids in membrane lipids is altered have been studied in a number of organisms. Most extensively investigated organism is Escherichia coli [40, 41]. Primary site of temperature control is elongation of $\Delta 9\text{-}C_{16:1}$ to $\Delta 11\text{-}C_{18:1}$. This is catalyzed by β-ketoacyl-ACP synthetase II. The mutant strain which lacks this enzyme cannot control the content of unsaturated fatty acids. Furthermore, much higher proportion of unsaturated fatty acids are synthesized upon 10°C by cells grown at 40°C (40°C-cells) than cells grown at 10°C (10°C-cells). This results suggest that fatty acid synthetase of different properties in 10°C-cells and 40°C-cells. Composition of fatty acids produced from fatty acid synthetase is solely determined by reaction temperature rather than growth temperature in B. ammoniagenes [42].

Proportion of unsaturated fatty acid is regulated by the level of fatty acid desaturase in B.megaterium [43]. When this bacterium grows at 35°C, transcription of the $C_{16:0}$ desaturase is blocked, and thus the desaturation do not occur. But when it grows at 20°C, the transcription occurs, and $C_{16:0}$ in phospholipid is desaturated.

In this paper, three topics are concerned:

(1) A psychrotrophic strict aerobe, Pseudomonas sp. strain E-3, posses both of the aerobic and the anaerobic pathways for the biosynthesis of unsaturated fatty acids, however the former pathway alone can sustain the

normal growth.

(2) Besides de novo synthesized fatty acids, exogenous fatty acid are utilized for phospholipids synthesis with and without modification (desaturation and elongation). Fatty acid metabolism was controlled according to the fatty acid supplemented.

(3) Some enzymatic characterization of fatty acid synthetase, fatty acid desaturase, and fatty acid elongase were performed.

Materials and Methods

Bacterial strains and culture media - Pseudomonas sp. strain E-3 (Pseudomonas E-3) was isolated in this laboratory from dust in a cold-room [44]. It is gram-stain negative, catalase positive, non spore forming [44], and strict aerobe. Pseudomonas E-3 belongs to psychrotroph, since it grow at 0-30^{o}C and optimum temperature for growth is 25^{o}C (Fig. 3). The bacterium was grown at the desired temperatures with vigorous shaking in Tris-salt medium (pH 7.2) consisting of 50 mM Tris-HCl, 50 mM NaCl, 10 mM KCl, 3.3 mM K_2HPO_4, 3 mM sodium citrate, 25 mM NH_4Cl, 1 mM $MgCl_2$, and 0.1 mM $FeSO_4$. 0.1 M sodium succinate and/or 0.04 % fatty acid (w/v) were used as the carbon source. When fatty acid was supplemented, 0.5 % (w/v) of Triton X-100 was added to the medium to disperse it. This concentration of the detergent did not affect the cell growth and fatty acid composition of membrane lipids.

Escherichia coli K-12 was grown in nutrient broth (peptone 10 g, meat extract 10 g, NaCl 5 g, and 1 l tap water, pH 7.2) at 37°C.

Lipids extraction and analysis - Cells in the late-logarithmic phase were harvested and washed once with deionized water by centrifugation at 3,000 x g for 10 min at 4°C. Lipids were extracted from the cells with chloroform and methanol by the method of Bligh and Dyer [45]. Phospholipids and neutral lipids were separated by silicic-acid column chromatography [46]. Amounts of total lipids, phospholipids, and neutral lipids were determined gravimetrically. Phospholipids were separated by TLC on silica gel plate using chloroform / methanol / acetic acid / water (65:25:0.5:3.5, v/v) as a solvent system. Each spot of phospholipids was scraped off and extracted three times with chloroform / methanol (1:1, v/v). They were quantified from phosphorous content by the method of Bartlett [47]. Neutral lipids were separated by TLC on silica gel using hexane / diethyl ether / acetic acid (8:2:0.5, v/v) as a solvent system. They were tentatively identified by comparing Rf value with standard sample.

Analysis of fatty acid composition - Lipids were transmethylated by heating in 5 % methanolic HCl at 90°C for 3 h. When lipid was separated, it was directly transmethylated along with silica gel. Fatty acid methyl esters were extracted three times with hexane, and analyzed by gas-liquid chromatography on a 2 m column of 10 % EGSS-X at 90-190°C (heating rate 4°C/min) with N_2 as the carrier

gas at flow rate of 30 ml/min.

Incorporation of radioactive materials into cellular fatty acids - To the 10 ml of middle-logarithmic phase cell culture grown at 15°C was added radioactive fatty acids at the desired concentration for each experiment, and incubated at 15°C for 1 h with shaking. Then, the culture was chilled in ice water, and the cells were harvested and washed once with chilled deionized water by centrifugation at 3,000 x g for 10 min at 4°C. For the determination of released $^{14}CO_2$, 50 ml/min of air was flowed through the culture and was bubbled into 5 ml of Hyamine 10X during the incubation. Then, 0.3 ml of the Hyamine 10X and 5 ml of scintillation cocktail (2,5-diphenyloxazole 4 g, 2,2'-phenylenebis (5'-phenyloxazole) 50 mg, toluene 1 l) were mixed in a scintillation vial, and the radioactivity was measured by liquid-scintillation counter. To make anaerobic conditions, the reaction mixture was flushed with N_2 gas for 15 to 30 min. To certify the anaerobic condition, one drop of reduced methylene blue was added to the medium. As long as anaerobic condition is maintained, the indicator remain colorless. Presence of trace amount of oxygen is indicated by expression of blue color.

Radioactive fatty acids were extracted and transmethylated by the same method as described above. The distribution of radioactivity among fatty acids was analyzed by radio gas-liquid chromatography or silver nitrate TLC. Each analytical condition was described in legends of figure and table.

Analysis of ^{14}C distribution in a radioactive fatty acid - Chemical cleavage of unsaturated fatty acid between its double bond was performed by permanganate-periodate oxidation [48]. Resulting monocarboxylic and dicarboxylic acid fragments were transmethylated with diazomethane and analyzed by radio gas-liquid chromatography.

Percentage of carboxyl carbon labeling was determined by the chemical decarboxylation [49].

Preparation and assay of fatty acid synthesizing system - Cells grown on succinate were sonically disrupted in 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-mercaptoethanol (buffer A). Unbroken cells and cell debris were removed by centrifugation at 10,000 x g for 20 min. The supernatant was centrifuged at 100,000 x g for 90 min. The supernatant of the second centrifugation was fractionated with 55-75 % saturation of ammonium sulfate and resolved in appropriate volume of buffer A. Then, the obtained sample was dialyzed. In some experiments, this fraction was used as fatty acid synthetase. In other instances, this preparation was stirred with activated charcoal for 2 h to remove low molecular substances. Fatty acid synthetase preparation was obtained by removing the charcoal by centrifugation. These preparations synthesized long chain fatty acids from acetyl-CoA and [2-^{14}C]malonyl-CoA. All the above procedures were carried out at 4^{o}C. ACP was prepared from E.coli K-12 according to the method of Rock and Cronan [50]. ACP from Pseudomonas E-3 was prepared from the supernatant of 75 % ammonium sulfate precipitation

of the soluble fraction. The supernatant was acidified to pH 3.9 by adding gracial acetic acid and stood for overnight at room temperature. The precipitate was collected by centrifugation at 8,000 x g for 10 min and suspended in appropriate volume of 50 mM sodium phosphate buffer (pH 7.0). The precipitate was dissolved by neutralization with 1 M Tris base. The solution thus obtained was used as the ACP of Pseudomonas E-3.

Protein concentration was determined by the method of Bradford using bovine γ-globulin as the standard [51].

The reaction mixtures (total volume 500 µl) contained varying concentration of acetyl-CoA, [2-^{14}C]malonyl-CoA (1.8 GBq/mmol), NADPH, and NADH, according to the experimental design. Besides them, 40 µg ACP, 140 µg enzyme protein, and buffer A were always included in the mixture. In some instances, NADPH and NADH regeneration systems instead of the above concentration of the reduced pyridine nucleotides were included in the reaction mixture; i.e. 1.5 mM NADP, 0.7 units glucose-6-phosphate dehydrogenase, 5 mM glucose-6-phosphate, 2 mM NADH, 2 units alcohol dehydrogenase, and 5 mM ethanol. The reaction was initiated by the addition of enzyme protein. After 1 h of incubation at 15 ^{o}C, fatty acid synthesis was terminated by the addition of 500 µl of 10 % methanolic KOH. The mixture was heated at 80^{o}C for 30 min. After acidification of the mixture to pH 3.0 with 5 N H_2SO_4, free fatty acids were extracted with diethyl ether. The radiolabeled fatty acids and carrier lipid (Pseudomonas E-3 total lipids) were transmethylated. Distribution of

radioactivity was determined by radio gas-liquid chromatography.

Preparation of membrane fraction and assay of fatty acid desaturase - Cells were suspended in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.25 M sucrose and were sonically disrupted for 2 min in an ice bath. The obtained suspension was used as crude cell extract. Unbroken cells and cell debris were removed by centrifugation (10,000 x g, 20 min). The supernatant was centrifuged again at 100,000 x g for 90 min. The precipitate was suspended in the same buffer to give a protein concentration approximately 10 mg/ml. This fraction was used as the membrane fraction. All procedures were done at 4°C.

Fatty acid desaturase was assayed with the following reaction mixture: 0.1 M sodium phosphate buffer (pH 7.0), 100 μM NADPH, 100 μM [1-^{14}C]$C_{16:0}$-CoA (1.9 MBq/μmol), and 0.3-1.0 mg of the membrane fraction in a total volume of 500 μl. The membrane fraction and mixture of other components were separately equilibrated to the desired reaction temperature. The reaction was initiated by mixing all the component and performed for 4 min. The following procedures of saponification, and transmethylation were the same as the described in fatty acid synthesizing system. Methyl esters of saturated and unsaturated fatty acids were separated by silver nitrate TLC. The separated spots were scraped off and were took into scintillation vial. Radioactivity was measured with a liquid-scintillation counter. Specific activity of the fatty acid desaturase was calculated by the

following formula.

$$\text{Formed unsaturated fatty acid /protein /min (nmol/mg protein/min)} = \frac{\text{radioactivity in unsaturated fatty acids}}{\text{radioactivity in total fatty acids}} \times \frac{\text{added } [^{14}C]C_{16:0}\text{-CoA (nmol)}}{\text{membrane protein (mg) x reaction time (min)}}$$

Preparation of nonradiolabeled and radiolabeled acyl-CoA and acyl-ACP - $C_{16:0}$-CoA and $C_{16:1}$-CoA were synthesized chemically by the method of Bishop and Hajra [52] from CoA-SH, and $C_{16:0}$ and $C_{16:1}$, respectively. Radioactive acyl-CoA was synthesized from [1-^{14}C]$C_{16:0}$ or [1-^{14}C]$C_{18:0}$, and CoA with the same method. [1-^{14}C]$C_{16:0}$-ACP was synthesized enzymatically from [1-^{14}C]$C_{16:0}$ and ACP isolated from E.coli K-12 cells by the method of Rock and Cronan [53].

Preparation and purification of 3-decynoyl-NAC - 3-Decynoyl-NAC was synthesized from 3-decynoic acid and NAC according to the method of Kass and Brock [54]. 3-Decynoic acid was obtained by oxidation of commercially obtained 3-decyn-1-ol [55] and converted to chloride salt [54]. NAC was synthesized by acetylation of cysteamine [56] and converted to a lead salt [54]. 3-Decynoic acid chloride and lead salt of NAC were combined by refluxing them in benzene at 81^{o}C for 1 h. Obtained 3-decynoyl-NAC was purified by reverse phase high performance liquid chromatography equipped with a TSK ODS-120T (4.6 mm x 250 mm) column using acetonitrile / water (9:1, v/v) as the solvent system at flow rate 1 ml / min. Concentration of

the product was quantified by measuring absorbance at 233 nm and calculated by using extinction coefficient of 4,000 [54].

Results

Lipid composition - Total lipids consisted of 90 % phospholipids and 10 % neutral lipids (Table 1). Phospholipids were phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (Table 1). Though total lipid content increased a little at 5°C, the proportion did not vary with alteration of growth temperature (Table 1). Based on co-chromatography with standard samples, neutral lipids were presumed to be free fatty acid, triacylglycerol, monoacylglycerol, and diacylglycerol.

Fatty acids composition - $C_{16:0}$, $C_{16:1}$, and $C_{18:1}$ were major fatty acids (Table 2). At 30°C, minor components increased a little. As the growth temperature lowered, $C_{16:1}$ and $C_{18:1}$ increased and $C_{16:0}$ decreased (Table 2). This response to temperature was more conspicuous in phosphatidylethanolamine, than in phosphatidylglycerol and cardiolipin (Table 3).

Position of double bond in $C_{16:1}$ and $C_{18:1}$ - Position of double bond in a unsaturated fatty acid molecule is prerequisite to determine the biosynthetic pathway by which they are synthesized. The double bond positions of $C_{16:1}$ and $C_{18:1}$ in Pseudomonas E-3 were determined by gas-liquid chromatography-mass spectrometry. Analytical results show that $C_{16:1}$ had fragment ions of m/z 73, 187, and 259, and

$C_{18:1}$ had fragment ions of m/z 73, 187, 287 (Fig. 4). These results indicate that $C_{16:1}$ and $C_{18:1}$ are Δ9-$C_{16:1}$ and Δ11-$C_{18:1}$, respectively.

Incorporation of radioactive fatty acids - When [1-^{14}C]$C_{16:0}$ was added to cell culture, 20 % of the added radioactivity was incorporated into cellular lipids, and 20 % of the incorporated radioactivity was found in unsaturated fatty acids (Table 4). Radioactivity was incorporated into phospholipids (90 %), neutral lipids (7 %), and water soluble fraction (3 %) after 30 min (Fig. 5). Incorporation of the radioactivity into lipids and the formation of [^{14}C]unsaturated fatty acid proceeded for initial 15 min and then leveled off (Fig. 5). A portion of the [1-^{14}C]$C_{16:0}$ was oxidized to form $^{14}CO_2$ (Table 4). Under the anaerobic conditions, no [^{14}C]unsaturated fatty acid was produced from [1-^{14}C]$C_{16:0}$ (Table 4). These results suggest that [1-^{14}C]$C_{16:0}$ was aerobically desaturated (the aerobic pathway). However, [^{14}C]unsaturated fatty acid can be obtained by β-oxidation followed by resynthesis through the anaerobic pathway. To examine this possibility, the same incorporation experiment was carried out in the presence of cerulenin. Cerulenin is a specific inhibitor of fatty acid synthesis [57]. When cerulenin (100 μg/ml) was added to the culture medium, radioactivity incorporated into fatty acids from [^{3}H]acetate reduced to 7 % of the control value (Table 4). The same concentration of cerulenin did not inhibit the formation of [^{14}C]$C_{16:1}$ from [1-^{14}C]$C_{16:0}$ (Table 4). Therefore, degradation and resynthesis of $C_{16:0}$ do not

participate to the conversion of $C_{16:0}$ to $C_{16:1}$. On the other hand, formation of [^{14}C]$C_{18:1}$ from [^{14}C]$C_{16:0}$ was inhibited by cerulenin (Table 4).

Another evidence to confirm the aerobic pathway is distribution of ^{14}C in a [^{14}C]$C_{16:1}$ molecule which was derived from [1-^{14}C]$C_{16:0}$. Exclusive distributions of ^{14}C on carboxyl end of $C_{16:0}$ and $C_{16:1}$ (Table 5) indicate that the [^{14}C]$C_{16:0}$ was directly desaturated to [^{14}C]$C_{16:1}$. If the [^{14}C]fatty acids are produced through degradation and resynthesis, distribution of ^{14}C would be randomized. For example, in [^{14}C]$C_{18:1}$ which was produced from [1-^{14}C]acetate, only 17 % of the radioactivity was on carboxyl end (Table 5). This value agree with the theoretically expected value (2/18 = 0.11).

When [^{3}H] or [^{14}C]acetate was radioactive precursor, 53 % of the radioactive fatty acids were unsaturated fatty acids (Table 4). To examine the participation of the anaerobic pathway to unsaturated fatty acid synthesis in vivo, the same experiment was carried out under the anaerobic conditions. Under the anaerobic conditions, radioactivity incorporated into fatty acids from [^{14}C]acetate reduced to 1.9 % of the value obtained under the aerobic conditions (Table 4). And the proportion of $C_{16:1}$ was severely reduced, whereas considerable radioactivity was incorporated into $C_{18:1}$ (Table 4). Thus, it is considered that $C_{16:1}$ is produced by the aerobic desaturation, and direct step of $C_{18:1}$ formation is chain elongation of pre-existing $C_{16:1}$.

When [1-^{14}C]$C_{12:0}$ was used as the radioactive precursor, radioactive $C_{16:0}$, $C_{16:1}$, and $C_{18:1}$ were appeared in cellular lipids (Table 4). No [^{14}C]$C_{12:0}$ was appeared in cellular lipids. Therefore, the added [1-^{14}C]$C_{12:0}$ was converted to longer saturated and unsaturated fatty acids, but was not directly esterified to phospholipids. This is another evidence of presence of the aerobic pathway in Pseudomonas E-3, because unsaturated fatty acid cannot be produced from saturated fatty acid longer than C_{10} by the anaerobic pathway (Fig. 2). The [^{14}C]$C_{16:1}$ derived from [1-^{14}C]$C_{12:0}$ was offered to chemical cleavage between its double bond. The result is shown in Fig. 6. The exclusive distribution of radioactivity on the fragment derived from carboxyl side confirms that the [^{14}C]$C_{16:1}$ is produced by elongation of [1-^{14}C]$C_{12:0}$ without degradation. When [1-^{14}C]$C_{14:0}$ was added to the culture medium, $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, and $C_{18:1}$ were radiolabeled (Table 4). This is also indicative for the operation of the aerobic pathway. And a portion of incorporated [^{14}C]$C_{14:0}$ was immediately esterified to phospholipids.

When [1-^{14}C]$C_{18:0}$ was radioactive precursor, much C_{18} and little C_{16} were radiolabeled (Table 4). The [^{14}C]$C_{18:1}$ was found to be mixture of Δ9-$C_{18:1}$ and Δ11-$C_{18:1}$ by silver nitrate TLC (Fig. 7A). Since Δ9-$C_{18:1}$ is not synthesized in Pseudomonas E-3 from acetate, it is expected to be synthesized by desaturation of $C_{18:0}$. This inference is supported by the finding that in vitro stearyl-CoA desaturation produced Δ9-$C_{18:1}$ (Fig. 7C). The [^{14}C]

Δ11-$C_{18:1}$ may be synthesized by condensation of $C_{16:1}$ and $[^{14}C]C_2$, since it was not appeared when cerulenin was added to the culture medium (Fig. 7B).

Effect of carbon source on the fatty acid composition in phospholipids - Pseudomonas E-3 is able to grow in the medium containing various fatty acid and hydrocarbon as a sole carbon source. Fatty acid composition in phospholipids was altered according to the kind of the carbon source. Cells grown on $C_{16:0}$ contained higher proportion of $C_{16:0}$ and $C_{16:1}$ in their phospholipids than the cells grown on succinate (Table 6). This suggests that the exogenous $C_{16:0}$ was directly transacylated to phospholipids and was also desaturated to $C_{16:1}$ before transacylation. Pseudomonas E-3 grew more slowly on this carbon source than on succinate (Fig. 8). When hexadecane was the sole carbon source, proportion of $C_{16:0}$ and $C_{16:1}$ was increased in phospholipids, too (Table 6). However, growth rate was lower than that on $C_{16:0}$ (Fig. 8). When $C_{16:1}$ was used as a carbon source, proportion of $C_{16:1}$ was increased in phospholipids (Table 6), and the growth rate was higher than the value obtained with the $C_{16:0}$-culture (Fig. 8).

When succinate was the carbon source, more than 98 % of the cellular fatty acids were even-chain at 15°C (Table 2). On the other hand, when $C_{11:0}$ or $C_{15:0}$ was used as the sole carbon source, saturated and unsaturated C_{15} and C_{17} fatty acids appeared in phospholipids (Table 6). It is most unlikely that the supplemented odd-chain fatty acid was cleaved to C_3 and C_2 and resynthesized to C_{15} and C_{17} fatty

acids, since propionyl(C_3)-CoA is converted to succinyl(C_4)-CoA [58]. When propionic acid was used as the carbon source, odd-chain fatty acids were only 14 % of the total fatty acids (Table 6). Thus, at least most of the odd-chain fatty acids were produced by elongation and aerobic desaturation of the supplemented $C_{11:0}$ or $C_{15:0}$. Conversely, the even-chain fatty acids produced are may be product of degradation followed by resynthesis.

When $C_{18:0}$ was sole carbon source, it was directly incorporated into phospholipids (Table 6), though it was scarcely detected in phospholipids in the cells grown on succinate (Table 2). When $\triangle 9$-$C_{18:1}$ or $C_{18:2}$ was carbon source, they were directly incorporated into phospholipids, though they were not synthesized in the cells grown on succinate. Even when the carbon source was C_{18} fatty acid, C_{16} fatty acids were major component in phospholipids (Table 6).

Effect of carbon source on the activity of fatty acid desaturation and composition of de novo synthesized fatty acids - Carbon source also affected to the activity of fatty acid desaturation and composition of de novo synthesized fatty acids. As shown in Table 7, $C_{16:0}$-desaturase activity was higher in the cells grown on $C_{16:0}$ or hexadecane than control (succinate-grown cells), but it was low in the cells grown on $C_{18:0}$ or unsaturated fatty acids. Furthermore, unsaturated fatty acids but not $C_{16:0}$ were synthesized from [^{14}C]acetate in $C_{16:0}$-grown cells (Table 8). Contrarily, in the cells grown on $C_{18:1}$,

proportion of [^{14}C]$C_{16:0}$ in de novo synthesized fatty acids was higher than the cells grown on succinate (Table 8).

Effects of growth and reaction temperatures on the desaturation activity - In vivo $C_{16:0}$-desaturation activity was affected by growth temperature of the cells and the assay temperature for the desaturation reaction. $C_{16:0}$-desaturation activity of the cells grown at 25°C (25°C-cells) was enhanced about 2.5-fold by lowering the assay temperature from 30°C to 5°C, whereas those of the cells grown at 5°C (5°C-cells) decreased a little at lower temperature (Fig. 9). At 5°C, the desaturation activity of 5°C-cells was only 10 % of that of 25°C-cells.

In vitro $C_{16:0}$ desaturase system - Above experiments using intact cells revealed that exogenous $C_{16:0}$ was desaturated by the aerobic pathway. The desaturase activity was assayed with cell free systems. As shown in Table 9, when [^{14}C]$C_{16:0}$-CoA was aerobically incubated with the membrane fraction in the presence of NADPH, [^{14}C]unsaturated fatty acid was produced. NADH could partly substitute for NADPH. Unesterified [^{14}C]$C_{16:0}$ was not desaturated by the membrane fraction nor the soluble fraction, but was desaturated when [^{14}C]$C_{16:0}$ was incubated with combination of the membrane fraction and concentrated soluble fraction in the presence of CoA, ATP, Mg^{2+}, and NADPH (Table 9). This results suggests that [1-^{14}C]$C_{16:0}$ was desaturated when is was esterified to form $C_{16:0}$-CoA by acyl-CoA synthetase in the soluble fraction. When [1-^{14}C]$C_{16:0}$-ACP was incubated with the membrane fraction,

radioactivities were detected in saturated fatty acids of phospholipids, and free fatty acids (Table 10). However, no radioactivity more than background level was found in unsaturated fatty acid. By the incubation of [^{14}C]$C_{16:0}$-ACP with soluble fraction, no radiolabeled unsaturated fatty acid was formed (data not shown). Another possibility that phospholipid is the substrate of desaturation might be excluded by considering the kinetics of incorporation and desaturation. As seen in Fig. 5, proportion of [^{14}C]unsaturated fatty acid increased as far as incorporation of radioactivity from [1-^{14}C]$_{16:0}$ into phospholipids continued, but it did not increase after the incorporation level off (Fig. 5). If phospholipid is desaturated, [^{14}C]unsaturated fatty acid would be increased even after the radioactivity incorporation leveled off. Therefore, it is concluded that [^{14}C]$C_{16:0}$ is desaturated before incorporation into phospholipids. Furthermore, time course of <u>in vitro</u> experiments with [1-^{14}C]$C_{16:0}$ supports this conclusion. Until the $C_{16:0}$-CoA was depleted, the [^{14}C]unsaturated fatty acid steadily increased (Fig. 10). However, after 4 min, as the [^{14}C]$C_{16:0}$-CoA reduced, the increasing rate of [^{14}C]unsaturated fatty acid slowed (Fig. 10).

When the [1-^{14}C]$C_{18:0}$-CoA was incubated with the membrane fraction, [^{14}C] Δ 9-$C_{18:1}$ was formed (Fig. 7). Therefore, <u>Pseudomonas</u> E-3 is able to desaturate $C_{18:0}$, if it is supplied in the form of $C_{18:0}$-CoA.

<u>Properties of partially purified fatty acid synthetase</u>

- Fatty acid synthetases in animals and plants, which have the aerobic pathway, produce only saturated fatty acids. To examine whether Pseudomonas E-3 follows this general rule, products of partially purified fatty acid synthetase was analyzed. Since this enzyme required ACP for full activity (Table 11), it is presumed to be type II fatty acid synthetase. Fatty acids were scarcely produced in the absence of NADPH, whereas 75 % of the full activity was obtained without NADH (Table 11). Incorportion of [2-^{14}C]malonyl-CoA into fatty acids proceeded linearly for 50 min at 15^{o}C. The ratio of unsaturated to saturated fatty acids did not change significantly with incubation time (Fig. 11).

Although varying the reaction conditions altered composition of products, both of saturated and unsaturated fatty acids were always produced by partially purified fatty acid synthetase (Table 12). When fatty acid synthesis was carried out under the anaerobic conditions, the same ratio of unsaturated to saturated fatty acids as that obtained under the aerobic conditions was produced (data not shown). Reaction conditions which were found to affect the product distribution are: (i) treatment of the enzyme with charcoal at the final step of the enzyme preparation; (ii) Mode of addition of NADPH and NADH. When NADPH and NADH were continuously supplied from their regeneration system, fatty acid synthetase without charcoal treatment produced only $C_{16:0}$ and $C_{18:1}$ (Table 12 a, g, h). However, after the treatment of the enzyme with charcoal, C_{10} - C_{18} fatty acids

were produced (Table 12 b). When fixed concentration of NADPH and NADH was added, C_{10} - C_{18} fatty acids were produced (Table 12 c, d, e, f). By omission of NADPH, average chain length decreased (Table 12 f); (iii) concentration of acetyl-CoA and malonyl-CoA (Table 12 g, h); and (iv) reaction temperature (Fig. 12). When ACP of Pseudomonas E-3 was used instead of E.coli ACP, both saturated and unsaturated fatty acids were produced, too (Table 12 e).

Effect of 3-decynoyl-NAC on fatty acid synthesis - Above results indicate that both the aerobic and the anaerobic pathways are present in Pseudomonas E-3. However, it is not clear what extent each pathway contribute to unsaturated fatty acid synthesis in vivo. Therefore, to assess the physiological role of the two pathways, effects of 3-decynoyl-NAC on the fatty acid synthesis and cell growth were examined.

As shown in Table 13, ratio of unsaturated to saturated fatty acids produced from [^{14}C]acetate by whole cells was scarcely affected by 3-decynoyl-NAC. However, total amount of synthesized fatty acids was reduced as the concentration of 3-decynoyl-NAC increased (Table 13). On the other hand, 3-decynoyl-NAC completely inhibited the unsaturated fatty acid synthesis by fatty acid synthetase (Table 13). Similarly to whole cells, total fatty acid synthesis by fatty acid synthetase was inhibited by 3-decynoyl-NAC (Table 13). Incorporation of [1-^{14}C]$C_{16:0}$ into cellular phospholipids and desaturation of [1-^{14}C]$C_{16:0}$ were not

affected by 3-decynoyl-NAC (Table 14). These results suggest that the aerobic pathway in whole cells was not inhibited by 3-decynoyl-NAC, though the anaerobic pathway was inhibited. To confirm this possibility, the effect of the inhibitor was examined with reconstituted system which contains partially purified fatty acid synthetase and membrane fraction. The later fraction contains fatty acid desaturase. As shown in Table 15, saturated and unsaturated fatty acids were produced from acetate irrespective to the presence of 3-decynoyl-NAC. Besides them, polar fatty acid, which was not migrate on silver nitrate TLC, was produced. This polar fatty acid did not migrate on plain TLC, too. However, after trimethylsililation, this component migrated as saturated fatty acid on silver nitrate TLC. Thus, this is presumed to be dihydroxy fatty acid. Because of very little quantity, further characterization of this component have not been undertaken.

The insensitivity of unsaturated fatty acid synthesis to 3-decynoyl-NAC can also be brought on if 3-decynoyl-NAC is modified to inactive form by Pseudomonas E-3 cells. However such a inactivation is excluded, because after incubation of 3-decynoyl-NAC with Pseudomonas E-3 cells, the substance retain the ability to inhibit the synthesis of unsaturated fatty acid by E.coli (data not shown).

The growth of Pseudomonas E-3 cells was not affected by 1×10^{-5} M of 3-decynoyl-NAC, but was completely inhibited by 1×10^{-4} M of the inhibitor (Fig. 13). The

growth inhibition by 1 x 10^{-4} M of 3-decynoyl-NAC was relieved by supplementation of $C_{16:0}$ or $C_{18:1}$ (Fig. 13). However, when $C_{16:0}$ was supplemented, proportion of unsaturated fatty acids in phospholipids was lower than the value obtained in succinate grown cells, and the obtained growth rate was lower than the control (Fig. 13).

$C_{16:1}$ elongation activity of membrane fraction - Judging from their structure, $\triangle 11\text{-}C_{18:1}$ is elongation product of $\triangle 9\text{-}C_{16:1}$. Tracer experiments confirmed that this elongation occurred in the cells (Table 4). To distinguish the elongation system from the fatty acid synthetase, fatty acid synthesis was examined with cell free systems with and without primer ($C_{16:1}$-CoA). When crude cell extract was used, besides saturated and unsaturated fatty acids, marked proportion of polar fatty acid was produced (Fig. 14). This component was not synthesized in the whole cells (Table 6). Both of acetyl-CoA and malonyl-CoA were utilized for fatty acid synthesis (Table 17), but $C_{16:1}$-CoA stimulated acetyl-CoA incorporation into fatty acids, whereas malonyl-CoA incorporation was rather inhibited by the addition of $C_{16:1}$-CoA (Table 17). Therefore, the elongation reaction may prefer acetyl-CoA as the C_2 donor, and general fatty acid synthesis utilizing malonyl-CoA may be inhibited by $C_{16:1}$-CoA. The elongation activity was detected also with membrane fraction (Fig. 15). On the other hand, neither soluble fraction nor fatty acid synthetase elongated $C_{16:1}$-CoA (data not shown).

Discussion

Phospholipids composition of Pseudomonas E-3 was the same as that of many gram-negative mesophilic bacteria [14, 15]. Although the proportion of phospholipids remain unchanged with alteration of growth temperature, proportion of unsaturated fatty acids increased with lowering the growth temperature (Table 2). Marked increase of unsaturated fatty acid was observed in phosphatidyl-ethanolamine which comprised to 75 % of total phospholipids (Table 1), indicating that fluidity of bulk membrane lipids was controlled.

Unsaturated fatty acids of Pseudomonas E-3 are Δ9-$C_{16:1}$ and Δ11-$C_{18:1}$ (Fig. 4). Scheuerbrandt and Bloch analyzed positions of double bond in monounsaturated fatty acids of many microorganisms and showed that the organism which utilize the anaerobic pathway contains a series of homologous even-numbered unsaturated fatty acids in which double bonds are located between carbon atoms of 7th and 8th counting from the methyl end of the fatty acid molecule, i.e. Δ7-C_{14}, Δ9-C_{16}, and Δ11-C_{18}. If a double bond is introduced between the 7th and 8th carbon of C_{16} by aerobic desaturation and then the chain is lengthened by C_2, Δ11-$C_{18:1}$ would be obtained. Such a pathway have been confirmed in cellular slime mold [59], rat liver cell [60], and Kaki pulp [61]. Therefore, the presence of Δ11-$C_{18:1}$ do not certainly exclude operation of the aerobic pathway. In this bacterium, $C_{16:1}$-CoA elongation activity was found

to be present in the membrane fraction (Fig. 15). The stimulating effect of $C_{16:1}$-CoA (Table 17), products pattern (Fig. 14, 15), and membrane bound activity distinguish this elongation system from fatty acid synthetase.

The same unsaturated fatty acids are synthesized in E.coli and Pseudomonas E-3, but their proportion in total fatty acids and the modes of alteration in response to temperature change are different between the two bacteria. In E.coli, $\triangle$11-$C_{18:1}$ is the major unsaturated fatty acids, and its percentage increase with lowering the growth temperature, whereas proportion of $\triangle$9-$C_{16:1}$ remains constant [10]. On the other hand, $\triangle$9-$C_{16:1}$ is predominant in Pseudomonas E-3, and its content changes with alteration of temperature (Table 2). Since melting point of $\triangle$9-$C_{16:1}$ (-0.5 - 0.5°C) is lower than that of $\triangle$11-$C_{18:1}$ (14.5 - 15.5°C), Pseudomonas E-3 membrane abundant in $\triangle$9-$C_{16:1}$ would be able to keep the membrane lipids fluid at about 0°C. And from this mode of alteration, the step of $\triangle$9-$C_{16:1}$ formation is considered to be the major site of temperature control of fatty acid composition in Pseudomonas E-3.

Experimental results with in vitro systems revealed that fatty acid desaturase in this bacterium utilized acyl-CoA (Table 9), but did not desaturate acyl-ACP (Table 10) and phospholipids (Fig. 5). The desaturation product, $C_{16:1}$-CoA was elongated to form $C_{18:1}$-CoA (Fig. 15). These membranous enzyme systems are similar to the desaturase and elongase systems found in animal cells [28] in the point

that they utilize acyl-CoA. Furthermore, acetyl-CoA utilization rather than malonyl-CoA for the elongation (Table 17) may be responsible to preferential incorporation of C_2 unit to $C_{18:1}$ under the anaerobic conditions (Table 4, 5). Besides fatty acid desaturation and elongation, active side reactions such as transacylation, degradation, and hydroxylation are detected in the membrane fraction (Figure 10, 14). The in vitro desaturase activity may be underestimated because of the substrate deficiency. To clarify the properties of the membrane bound desaturation and elongation systems, identification of the polar fatty acid (Fig. 14, Table 15) and purification of the enzyme protein from the membrane fraction are necessary.

In vivo and in vitro experiments revealed that the aerobic pathway is operating in Pseudomonas E-3. On the other hand, fatty acid synthetase also produced unsaturated fatty acids (Table 12). This is first report of the co-existence of the aerobic and the anaerobic pathways in an organism. The two pathways distribute mutually exclusive in all organisms so far examined. The anaerobic pathways were found in strict and faculative anaerobic bacteria, and some aerobic bacteria while the aerobic pathways were discovered in some aerobic bacteria, blue-green algae, and all eukaryotes [39, 62, 63]. Considering these phylogenetic distribution, the co-existence of the two pathways may be intermediary in the evolution of the biosynthetic pathway of unsaturated fatty acids. Up to this time, genus Pseudomonas have been considered to have only the anaerobic pathways

[25, 38, 39, 62, 63], though it is strict aerobic bacteria. This conclusion is based on the experiments of some species in this genus. Since Pseudomonas is quite heterogenous group [64], and the aerobic pathway has physiological advantage for cell growth (see below discussion), deviation of Pseudomonas E-3 from the above general rule is not unacceptable. In general, gram-positive aerobes (mycobacteria, corynebacteria, bacilli, and micrococci) utilize the aerobic pathway, but gram-negative aerobes are unpredictable.

Other interests for the co-existence of the two pathways are their relative contribution and physiological meaning. So far examined, the anaerobic pathway seems not to work in vivo at least as major route for the biosynthesis of unsaturated fatty acids. Anaerobiosis from acetate resulted marked reduction of $C_{16:1}$ (Table 4). This result suggests that the aerobic pathway is concerned in the production of the most $C_{16:1}$. Anaerobic formation of $C_{18:1}$ (Table 4) can be explained by chain elongation of pre-existing $C_{16:1}$. 3-Decynoyl-NAC, inhibitor of the anaerobic pathway, did not affect the ratio of unsaturated to saturated fatty acids synthesized in whole cells, whereas completely blocked unsaturated fatty acid synthesis by fatty acid synthetase (Table 13). Sensitivity of total fatty acids synthesis to 3-decynoyl-NAC was similar in three different systems of Pseudomonas E-3, fatty acid synthetase, reconstituted system, and whole cells (Table 13, 15). These results indicate that 3-decynoyl-NAC effectively

inhibits the anaerobic pathway in _in vivo_ as well as _in vitro_ system. Thus, the aerobic pathway can produce enough unsaturated fatty acid to sustain the growth under the condition that the anaerobic pathway is inhibited.

The growth inhibition by 1×10^{-4} M of 3-decynoyl-NAC seems to be due to insufficiency of total fatty acids rather than inhibition of unsaturated fatty acid synthesis, because the ratio of unsaturated to saturated fatty acids newly synthesized was not altered (Table 13), and the total fatty acid synthesis is reduced to 12 % of the control by the addition of the inhibitor at this concentration (Table 13). Moreover, addition of $C_{16:0}$ relieved the growth inhibition (Fig. 13). This result indicates that a part of the supplemented $C_{16:0}$ was desaturated to $C_{16:1}$ by the aerobic pathway and used for membrane lipids synthesis. However, the addition of $C_{16:0}$ only partly recovered the cell growth, though the supplementation of $C_{18:1}$ almost fully restored the cell growth (Fig. 13). Proportion of unsaturated fatty acids in phospholipids (Table 16) cannot explain this difference. The relationship between the kind of carbon source, fatty acid composition of phospholipids, and cell growth rate is complicated. For example, when $C_{16:0}$ was carbon source, the growth rate is low than that of control (succinate-culture) (Fig. 8) in spite of its high content of unsaturated fatty acids (73 %, Table 6). Rather, it seems that cellular energy economy to obtain unsaturated fatty acids determine the growth rate. Conversely, when a unsaturated fatty acid (1.8 mM) was sole carbon source, cell

growth rate was similar to that obtained with 100 mM succinate as the carbon source (Fig. 8). Thus, the formation of membrane lipids utilizing exogenous unsaturated fatty acid is fairly advantageous for the growth at 15°C.

Pseudomonas E-3 cells synthesizes C_{16} and C_{18} fatty acids from acetate (Table 4). Products of fatty acid synthetase was not agree with this cellular composition. They contained shorter chain (C_{10} - C_{14}) fatty acids (Table 12). However, their chain length distribution and proportion of unsaturated to saturated fatty acids were greatly changed by the incubation conditions (Table 12). Thus, products of fatty acid synthetase may be controlled to suit the cellular requirement in vivo. Another possible control site of fatty acid composition is transacylation of fatty acid to phospholipids. For instance, exogenous $C_{11:0}$ or $C_{12:0}$ itself did not appeared in cellular phospholipids (Table 4, 6), whereas supplemented $C_{15:0}$, $C_{16:0}$, or C_{18}s were directly acylated to phospholipids (Table 4, 6). These chain length selectivity agree with the chain length of cellular components, i.e. C_{16} and C_{18}. However, $C_{14:0}$ was scarcely found in phospholipids of succinate-grown cells (Table 2), whereas it was rapidly incorporated into phospholipids when it was exogenously supplemented (Table 4). This discrepancy remains unsolved at present stage.

This bacterium utilizes exogenous long chain (C_{16} and C_{18}) fatty acid for phospholipids synthesis. For the de novo synthesis of membrane lipids, much energy and materials are necessary (16acetyl-CoA + 28NADPH + 28H^+ + 14ATP +

glycerol-3-phosphate ⟶ dipalmitoyl-phosphatidic acid + 28$NADP^+$ + 14ADP + 14Pi). Since economy of energy and nutrients is important for bacterial growth, the utilization of exogenous fatty acid for the phospholipids synthesis is reasonable. According to fatty acid supplementation, de novo fatty acid synthesis was altered so as to save the synthesis of the supplemented one (Table 8). And the activity of $C_{16:0}$ desaturation rose when hexadecane or $C_{16:0}$ was carbon source, whereas the activity decreased when $C_{18:0}$ or unsaturated fatty acid was carbon source (Table 7). As a result, proportion of $C_{16:0}$ and $C_{16:1}$ were increased in phospholipids of $C_{16:0}$-grown cells than succinate grown cells (Table 6). On the other hand, proportion of $C_{16:1}$ is lower in $C_{18:1}$- or $C_{18:2}$-grown cells than succinate-grown cells (Table 6). In these cells, the exogenous unsaturated fatty acid substituted to $C_{16:1}$.

Exogenous fatty acid was incorporated into phospholipids with and without modification. Saturated fatty acid was desaturated by the aerobic pathway. This conversion correspond with the necessity of unsaturated fatty acid for the growth at low temperature. In the organism in which only the anaerobic pathway operates, desaturation of exogenous saturated fatty acid is impossible. For example, in E.coli, exogenous fatty acid is oxidatively degraded to C_2 [65], to shorter chain fatty acid [66], or directly incorporated into complex lipids [67], but is not desaturated nor elongated [64]. For the desaturation of exogenous fatty acid, it is also prerequisite that the

substrate of desaturation is acyl-CoA. Transport of exogenous fatty acid is coupled with activation to acyl-CoA [65]. For example, exogenous $C_{16:0}$ or $C_{18:0}$ are converted to monounsaturated fatty acids in animal and yeast cells. Fatty acid desaturase in these organism utilize acyl-CoA [29, 68]. On the other hand, cells of higher plants cannot produce unsaturated fatty acid from exogenous $C_{16:0}$ or $C_{18:0}$ [69, 70]. In plant cells, exogenous $C_{16:0}$ or $C_{18:0}$ is acylated to acyl-CoA, but is not convert to acyl-ACP [71, 72]. Thus, acyl-ACP desaturase in plant cells cannot desaturate exogenous $C_{16:0}$ or $C_{18:0}$. In plant cells, endogenous $C_{16:0}$ or $C_{18:0}$-ACP produced from acetate is able to be desaturated.

The different response of the $C_{16:0}$-desaturase activity in the cells grown at 25°C (25°C-cells) and the cells grown at 5°C (5°C-cells) (Fig. 9) can be explained in relation to pre-existing fatty acid composition. Since the 5°C-cells already contains higher proportion of unsaturated fatty acids than that of 25°C-cells (Table 2), the desaturation activity is not requisite. On the other hand, for the 25°C-cells, which contains lesser proportion of unsaturated fatty acid, formation of unsaturated fatty acids is necessary to increase the unsaturated fatty acids in membrane lipids. However, since the substrate of desaturation is $C_{16:0}$-CoA, the enzyme cannot desaturate phospholipid-bound fatty acid. The acyl-CoA desaturase can modify only the newly synthesized or exogenously supplied fatty acids. However, if retailoring system operates,

modification of pre-existing fatty acids by acyl-CoA desaturase is possible, i.e. acyl chain is released from phospholipid, desaturated, and reacylated to lysolipid (monoacyl-phospholipid) [73]. Activity of the retailoring system is relatively high in eukaryotes [73], but is scarcely detected in E.coli [73]. Now, no information is available whether the retailoring system is operating or not in Pseudomonas E-3.

In the cells grown on succinate, $C_{18:0}$ was scarcely detected in phospholipids (Table 2). On the other hand, when [1-^{14}C]$C_{18:0}$ was added to the medium, it was directly incorporated into cellular lipids (Table 4). And in the cells grown on $C_{18:0}$, $C_{18:0}$ forms 13 per cent of total fatty acids in phospholipids (Table 6). These results indicate that $C_{18:0}$ is acylated to phospholipids if it is supplied in plenty. Therefore determinative step for the the presence or absence of $C_{18:0}$ in phospholipids is before transacylation.

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Table 1. Effect of growth temperature on lipids composition of *Pseudomonas* E-3

	Growth temperature		
	5°C	15°C	30°C
	(mg lipids / g cell dry weight)		
Total lipids	109.4 ± 4.2	78.4 ± 12.8	81.7 ± 5.6
Phospholipids	102.8 ± 3.9	68.6 ± 2.7	76.2 ± 6.9
Neutral lipids	6.6 ± 3.9	9.8 ± 2.7	5.5 ± 6.9
Phospholipids	(% of total phospholipids)		
PE[a]	74.7 ± 3.4	72.5 ± 2.3	79.0 ± 2.4
PG[b]	18.4 ± 4.4	19.3 ± 2.5	15.2 ± 1.7
CL[c]	6.9 ± 1.8	8.2 ± 0.4	5.8 ± 0.9

Values are average of 4 experiments ± S.D.

[a] Phosphatidylethanolamine. [b] Phosphatidylglycerol.

[c] Cardiolipin.

Table 2. Fatty acid composition of total lipids of *Pseudomonas* E-3

Fatty acid	Growth temperature		
	5°C	15°C	30°C
	mol %		
12:0	t[a]	0	t
12:1	t	0	t
14:0	0.5 ± 0.3	1.5 ± 1.2	1.7 ± 0.5
14:1	t	0	t
16:0	16.1 ± 1.1	20.3 ± 0.6	36.4 ± 1.7
16:1	59.4 ± 0.4	55.3 ± 1.9	45.2 ± 1.1
17:0	0	0.4 ± 1.0	1.8 ± 0.2
17:1	0	0.5 ± 0.4	1.6 ± 0.2
18:0	0	0.6 ± 0.2	2.8 ± 1.2
18:1	23.7 ± 1.2	20.8 ± 1.8	8.4 ± 0.7
N.D.[b]	0.4 ± 0.2	0.7 ± 0.0	2.1 ± 0.4

Fatty acids are denoted by the chain length : number of double bond. Values are averages of three different experiments ± S.D. [a] t, trace amount (below 0.4 %). [b] Not determined.

Table 3. F

	ipin	
Fatty acid	5°C	30°C
12:0	0	2.2 ± 1.4
14:0	0	2.6 ± 0.6
14:1	0	4.0 ± 5.9
16:0	20.8 ± 8	20.2 ± 5.0
16:1	57.0 ± 4	52.6 ± 6.3
17:0	01	t
17:1	0	t
18:0	06	2.1 ± 2.8
18:1	21.8 ± 7	16.3 ± 8.2

Fatty acids a
are averages
a t, trace amc

Table 3. Fatty acid composition of each phospholipid class

Fatty acid	Phosphatidylglycerol			Phosphatidylethanc		
				Growth temperat		
	5°C	15°C	30°C	5°C	15°C	
					mol %	
12:0	0	2.4±0.5	2.1±1.1	0	0.7±0.5	1
14:0	0	0.9±0.2	1.7±0.8	0	1.9±0.3	2
14:1	0	0	1.1±0.8	0	0	4
16:0	20.8±2.5	21.3±3.1	28.6±2.0	19.2±1.8	24.8±0.6	35
16:1	57.0±3.5	59.6±2.5	52.4±5.0	57.6±2.3	53.3±3.2	44
17:0	0	0	t[a]	0	0	
17:1	0	0	t	0	0	
18:0	0	1.4±0.6	2.3±2.0	0	1.4±1.3	3
18:1	21.8±3.8	14.4±3.0	11.8±2.8	23.2±2.9	17.9±2.6	8

Fatty acids are denoted by the chain length : number of double b
are averages of three different experiments ± S.D.
[a] t, trace amount (below 0.4 %).

spholipid class of *Pseudomonas* E-3

osphatidylethanolamine		Cardiolipin		
Growth temperature				
15°C	30°C	5°C	15°C	30°C
mol %				
0.7 ± 0.5	1.8 ± 2.5	0	0	2.2 ± 1.4
1.9 ± 0.3	2.9 ± 0.9	0	0	2.6 ± 0.6
0	4.5 ± 5.6	0	0	4.0 ± 5.9
.8 24.8 ± 0.6	35.6 ± 1.9	22.3 ± 5.5	17.2 ± 0.8	20.2 ± 5.0
.3 53.3 ± 3.2	44.1 ± 3.1	56.9 ± 2.9	57.4 ± 8.4	52.6 ± 6.3
0	t	0	1.1 ± 1.1	t
0	t	0	0	t
1.4 ± 1.3	3.1 ± 0.7	0	1.4 ± 1.6	2.1 ± 2.8
.9 17.9 ± 2.6	8.0 ± 3.4	20.8 ± 10.0	22.9 ± 6.7	16.3 ± 8.2

umber of double bond. Values
D.

Table 4

Labeled	on of radioactivity of total)		
	16:1	18:0	18:1
[1-^{14}C]	39	0	14
	4	0	34
[^{3}H]Ace	36	0	16
sod			
	0	0	27
	12	0	1
[1-^{14}C]	33	0	30
	3	0	0
[1-^{14}C]	36	0	16
[1-^{14}C]	18	0	2
	<1	0	<1
	18	0	0
[1-^{14}C]	7	65	26

[a] Numb

[b] Not

[c] For nditions, 1.67 nmol of r

Table 4. Distribution of radioactivity among fatty acids in total lipid

Labeled Precursor	Atmospheric condition	Amount added to culture (nmol/ml)	Cerulenin (μg/ml)	Percent of radioactiv in lipids
[1-^{14}C]Acetate sodium salt	Aerobic	0.20	0	27.0
	Anaerobic	0.20	0	0.5
[^{3}H]Acetate sodium salt	Aerobic	0.03	0	5.7
	Anaerobic	0.03	0	0.1
	Aerobic	0.03	100	0.1
[1-^{14}C]Lauric acid	Aerobic	0.19	0	1.2
	Anaerobic	0.19	0	—
[1-^{14}C]Myristic acid	Aerobic	0.19	0	21.8
[1-^{14}C]Palmitic acid	Aerobic	0.18	0	20.0
	Anaerobic	0.18	0	19.6
	Aerobic	0.18	100	21.6
[1-^{14}C]Stearic acid	Aerobic	0.17	0	3.2

[a] Number of carbons: number of double bond.

[b] Not determined.

[c] For the determination of radioactive fatty acids composition which we of radioactive acetate was added to 10 ml of the culture medium to ov

acids in total lipids of Pseudomonas E-3

rulenin ug/ml)	Percent of added radioactivity in lipids (%)	Percent of added radioactivity in lipids (%)	Distribution of radioactivity (% of total)				
			14:0[a]	16:0	16:1	18:0	18:1
0	27.0	18.6	0	48	39	0	14
0	0.5	—[b]	0	62[c]	4	0	34
0	5.7	—	0	48	36	0	16
0	0.1	—	0	73[c]	0	0	27
100	0.1	—	0	87	12	0	1
0	1.2	2.7	0	36	33	0	30
0	—	—	0	97	3	0	0
0	21.8	34.5	23	26	36	0	16
0	20.0	14.0	0	80	18	0	2
0	19.6	—	0	99	<1	0	<1
00	21.6	—	0	82	18	0	0
0	3.2	5.1	0	2	7	65	26

composition which were produced under the anaerobic conditions, 1.67 nmol
culture medium to overcome low incorporation.

Table 5. Carboxyl carbon labeling of fatty acids derived from [1-^{14}C]fatty acids.

Radiolabeled precursor	Atmospheric condition of incorporation	^{14}C in carboxyl carbon (% of total radioactivity) 16:0[a]	16:1	18:1
[1-^{14}C]Palmitic acid	Aerobic	101.5 ± 3.1[b]	99.7 ± 6.3	42.5 ± 3.0
[1-^{14}C]Acetate sodium salt	Aerobic	ND[c]	ND	16.9 ± 2.3
	Anaerobic	ND	ND	48.3 ± 8.1

[a] Number of carbons : number of double bond.

[b] Average of three or two experiments ± standard deviation.

[c] Not detected.

Table 6. Effect of carbon source on the fatty acid composition of total phospholipids

Fatty acid	Carbon source									
	C4[a]	C3[b]	11:0[c]	15:0	16:0	16:1	18:0	18:1	18:2	Hexa-decane
	Mol %									
14:0	tr[d]	0	0	0	tr	tr	tr	tr	4	1
15:0	0	3	12	28	0	0	0	0	0	0
15:1	0	1	6	7	0	0	0	0	0	0
16:0	21	17	4	4	26	18	20	20	19	26
16:1	51	45	19	17	59	59	47	38	35	50
17:0	0	4	12	9	0	0	0	0	0	1
17:1	tr	6	36	27	tr.	tr	1	2	3	tr
18:0	1	0	tr	0	tr	tr	13	1	0	1
18:1	25	24	8	8	14	20	16	39	25	21
18:2	0	0	0	0	0	0	0	0	11	0
N.D.[e]	2	0	3	0	1	3	3	0	3	0

Values are averages of two measurements. Deviations are within 3 %. [a] Succinate. [b] Propionate. [c] Number of carbons: number of double bond. [d] Trace amount (below 0.5 %). [e] Not determined.

Table 7. Effect of carbon cource on the fatty acid desaturation activity

Carbon source	Desaturation substrate	
	[1-^{14}C]16:0[a]	[1-^{14}C]18:0
	% of desaturation	
Succinate	20.8	33.0
16:0	27.5	-[b]
16:1	6.5	-
18:0	4.0	48.0
18.1	1.2	-
18:2	8.4	-
Hexadecane	27.8	-

[a] Number of carbons: number of double bond.

[b] Not determined.

Table 8. Effect of carbon cource on the composition of *de novo* synthesized fatty acids

Carbon source	Distribution of radioactivity among fatty acids produced from [1-^{14}C]acetate (% of radioactivity)		
	16:0[a]	16:1	18:1
Succinate	32	43	25
16:0	0	80	20
18:1	45	34	21

[a] Number of carbons: number of double bond.

Table 9. In vitro desaturase activity

System	Unsaturated fatty acid production (nmol/mg protein/minute)
[1-^{14}C]Palmityl-CoA as the substrate	
Complete system [a]	0.84
- NADPH	0.07
- NADPH, + NADH	0.62
Boiled membrane	0
Soluble fraction	0
Nitrogen atmosphere	0
[1-^{14}C]Palmitic acid as the substrate	
Membrane fraction supplemented with soluble fraction and cofactors [b]	0.51
- Soluble fraction	0

[a] Reaction mixture consisted of 100 μM [1-^{14}C]palmitoyl-CoA, 100 μM NADPH, 0.5-1.0 mg of membrane protein, and 0.1 M sodium phosphote buffer (pH 7.0) in total volume 500 μl. Reaction mixture was incubated at 25°C for 4 min.

[b] Reaction mixture consisted of NADPH 1 mM, ATP 10 μM, CoA 1 μM, DTT 5 μM, soluble fraction 280 μg protein, membrane fraction 50 ug protein, [1-^{14}C]palmitic acid 10 nmol (8.3×10^4 cpm), and 0.1 M sodium phosphate buffer (pH 7.0) in total volume 1.0 ml.

Table 10. Distribution of radioactivity in each lipid class from [1-^{14}C]palmitoyl-ACP incubated with membrane fraction

Lipid class	Radioactivity (cpm)	
	SFA[a]	UFA[b]
Phospholipids	14,820	85
Triacylglycerol	1,819	70
Fatty acids	7,037	82
Acyl-ACP	14,911	54

Reaction mixture consisted of 50 nmol [1-^{14}C]palmitoyl-ACP (47,000 cpm), 100 μM NADPH, 350 μg membrane protein, 0.1 M sodium phosphate buffer (pH 7.0), in total volume 500 μl. Incubation wascarried out at 15°C for 5 min. [a] Saturated fatty acid, [b]Unsaturated fatty acid.

Table 11. Cofactor requirement of partially purified fatty acid synthetase

Reaction system	Incorporation of malonyl-CoA (nmol/min/mg protein)
Complete system [a]	52.4
- acetyl-CoA	10.4
- NADPH	0.4
- NADH	39.1
- ACP	6.2
ACP of Pseudomonas E-3	25.0

[a] 50 μM acetyl-CoA, 20 μM [2-^{14}C]malonyl-CoA (55.8 Ci/mol), 900 μM NADPH, 900 μM NADH, 40 μg E.coli ACP, 140 μg enzyme protein, 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-mercaptoethanol, total volume 500 μl.

T f		a b c d e° f g h		N° g w

Table 12. Effects of varying incubation conditions on the products o
fatty acid synthetase

	Charcol treatment	Ace-CoA (μM)	Mal-CoA (μM)	NADPH (μM)	NADH (μM)	Distribution of (%)			
						10:0[a]	12:0	14:0	16:0
a	-	50	20	RS[b]	RS	0	0	0	30
b	+	50	20	RS	RS	8	11	46	11
c	-	50	20	900	900	15	7	21	25
d	+	50	20	900	900	4	2	16	28
e[c]	+	50	20	900	900	10	2	14	35
f	+	50	20	900	0	8	0	25	23
g	-	25	10	RS	RS	0	0	0	27
h	-	1,000	10	RS	RS	0	0	0	50

[a] Number of carbons : number of double bond. [b] Regeneration system, alc
glucose-6-phosphate dehydrogenase for NADPH. [c] ACP of Pseudomonas E-3
were carried out using E.coli ACP.

ns on the products of partially purified

Distribution of radioactivity (%)							Ave. chain length	UFA %
:0*	12:0	14:0	16:0	16:1	18:0	18:1		
0	0	0	30	0	0	70	17.4	70
8	11	46	11	21	0	3	15.7	24
5	7	21	25	16	0	16	14.7	32
4	2	16	28	24	15	11	15.9	35
0	2	14	35	17	12	10	15.5	27
8	0	25	23	28	5	11	15.3	39
0	0	0	27	0	0	73	17.5	73
0	0	0	50	0	0	50	17.0	50

neration system, alcohol dehydrogenase for NADH and
P of Pseudomonas E-3 was used. Other experiments

Table 13. Effect of 3-decynoyl-NAC on the fatty acid synthesis by *Pseudomonas* E-3 whole cells and fatty acid synthetase

Reaction system	Concentraation of 3-decynoyll-NAC (μM)	Radioactivity in total fatty acids (DPM)	Distribution of radioactivity (%) SFA[a]	UFA[b]
Whole cells	0	285,146(100)[c]	28	72
	1×10^{-5}	153,411(54)	30	70
	1×10^{-4}	33,620(12)	33	68
Fatty acid synthetase	0	70,837(100)	27	73
	1×10^{-5}	42,795(60)	100	0

[a] Saturated fatty acid. [b] Unsaturated fatty acid. [c] Values in parentheses are relatives to the value obtained in the absence of 3-decynoyl-NAC.

Table 14. Effect of 3-decynoyl-NAC on the desaturation and incorporation of [1-^{14}C]palmitic acid by whole cells

Concentration of 3-decynoyl-NAC (μM)	Radioactivity in total fatty acids (DPM)	Distribution of radioactivity (%) SFA[a]	UFA[b]
0	6.724	83	17
1×10^{-5}	6.950	85	15
1×10^{-4}	6.712	84	16

[a] Saturated fatty acid. [b] Unsaturated fatty acid.

Table 15. Effect of 3-decynoyl-NAC on the fatty acid synthesis in reconstituted system

Concentration of 3-decynoyl-NAC (μM)	Radioactivity in total fatty acids (DPM)	Distribution of radioactivity (%) SFA[a]	UFA[b]	PFA[c]
0	109,252(100)[d]	39.3	36.4	24.3
1×10^{-5}	68,062(62)	36.1	39.8	24.0
1×10^{-4}	18,168(17)	36.2	35.8	28.0

Reaction mixture consisted of membrane fraction (0.5 mg protein), partially purified fatty acid synthetase(1.0 mg protein), [1-^{14}C] acetate 170 nmol (2.2 GBq/mmol), ATP 100 nmol, NADPH 100 nmol, CoA 10 nmol, NADH 100 nmol, ACP 40μg protein, and 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-mercaptoethanol in total volume 1.0 ml. [a] Saturated fatty acid, [b] Unsaturated fatty acid. [c] Polar fatty acid. [d] Numbers in parentheses are relatives to the value obtained in the absence of 3-decynoyl-NAC.

Table 16. Effect of 3-decynoyl-NAC on the fatty acid composition of phospholipids in cells grown on succinate and fatty acid

Fatty acid	Control: Succinate medium	3-Decynoyl-NAC 0		3-Decynoyl-NAC 1×10^{-4} M	
		Fatty acid supplemented			
		16:0[a]	18:1	16:0	18:1
		Mol %			
14:0	tr[b]	tr	1	1	1
16:0	21	27	16	32	26
16:1	51	58	36	41	27
17:1	tr	tr	tr	2	1
18:0	1	tr	1	0	0
18:1	25	14	45	24	45
N.D.[c]	2	1	1	0	0

Growth medium consisted of 100 mM succinate, 0.5 % Triton X-100, 0.04 % fatty acid, and indicated concentration of 3-decynoyl-NAC.
[a] Number of carbons: number of double bond.
[b] Trace amount (below 0.5 %). [c] Not determined.

Table 17. Activity of fatty acid synthesis and chain elongation in crude cell extract

C2 donor	$C_{16:1}$-CoA	C2 donor incorporated into fatty acids (nmol/mg protein/15 min)
Acetyl-CA	−	0.15(100)[a]
	+	0.26(170)
Malonyl-CoA	−	1.01(100)
	+	0.70(69)

Reaction mixture consisted of crude cell extract (1 mg protein), NADPH 100 nmol, $C_{16:1}$-CoA 100 nmol, [2-^{14}C]malonyl-CoA or [1-^{14}C]acetyl-CoA 50 nmol, and 0.1M sodium phosphate buffer (pH 7.0) in total volume 1.0 ml. [a] Numbers in parentheses are relative values to the value obtained without $C_{16:1}$-CoA for each C2 donor.

Figure 1. Reaction steps of fatty acid synthesis

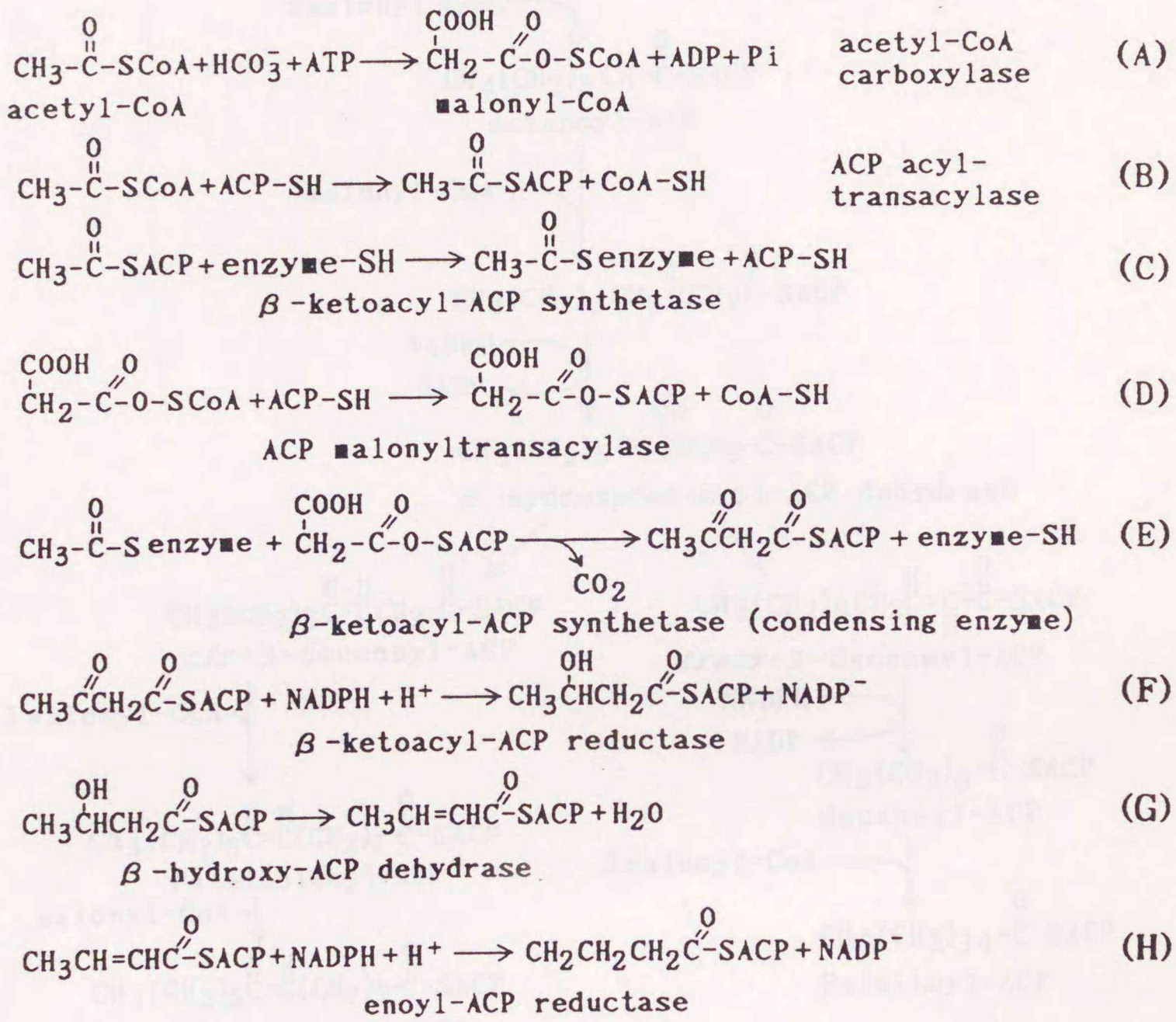
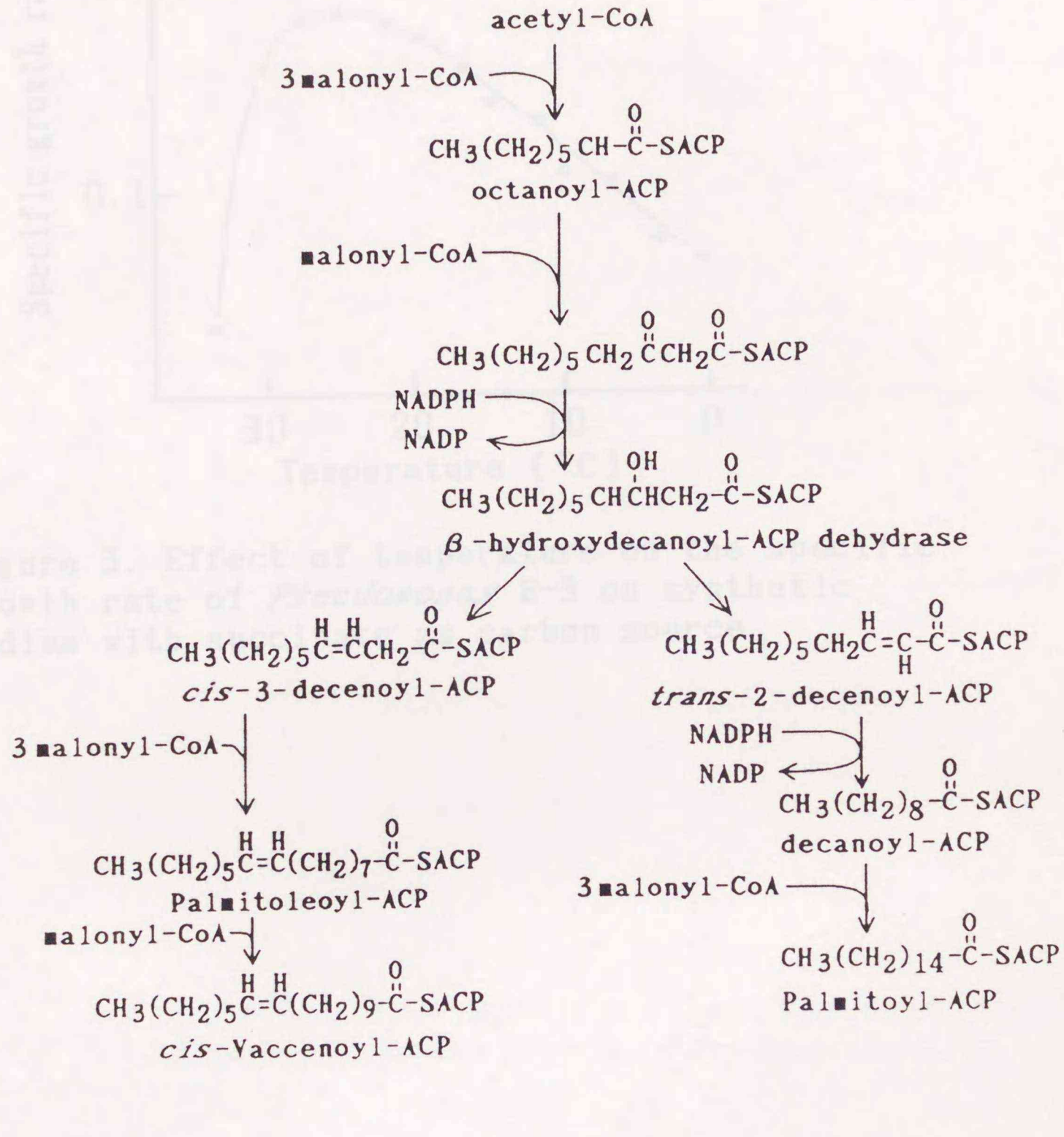


Figure 2. Anaerobic production of unsaturated fatty acids



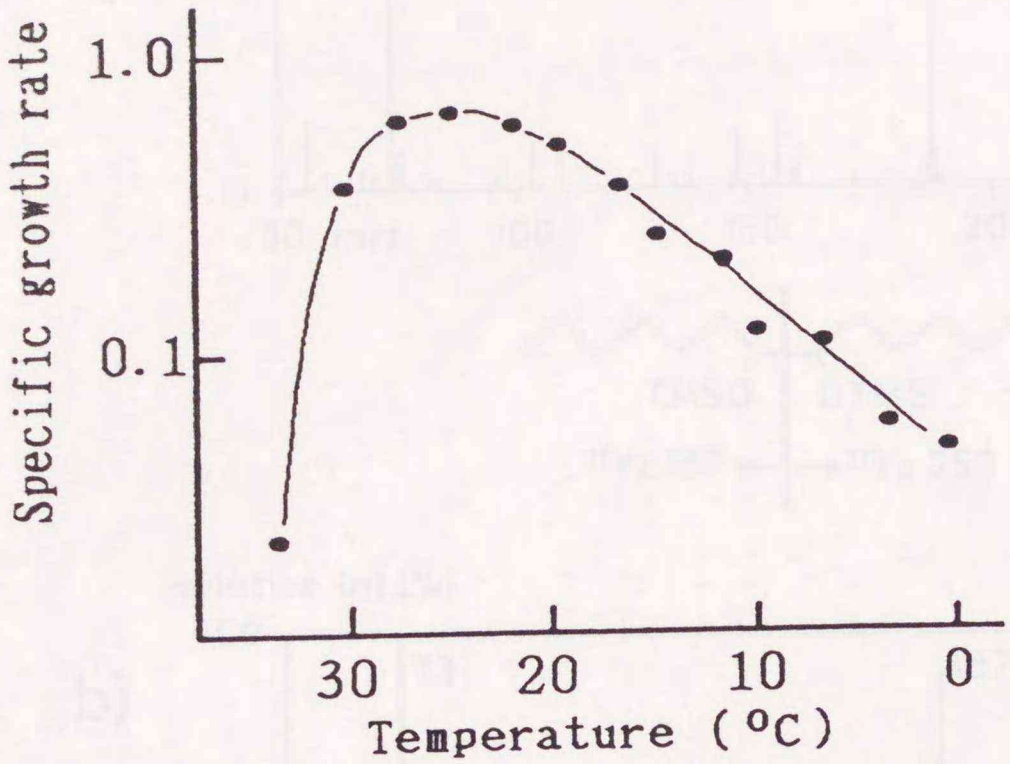


Figure 3. Effect of temperature on the specific growth rate of *Pseudomonas* E-3 on synthetic medium with succinate as carbon source.

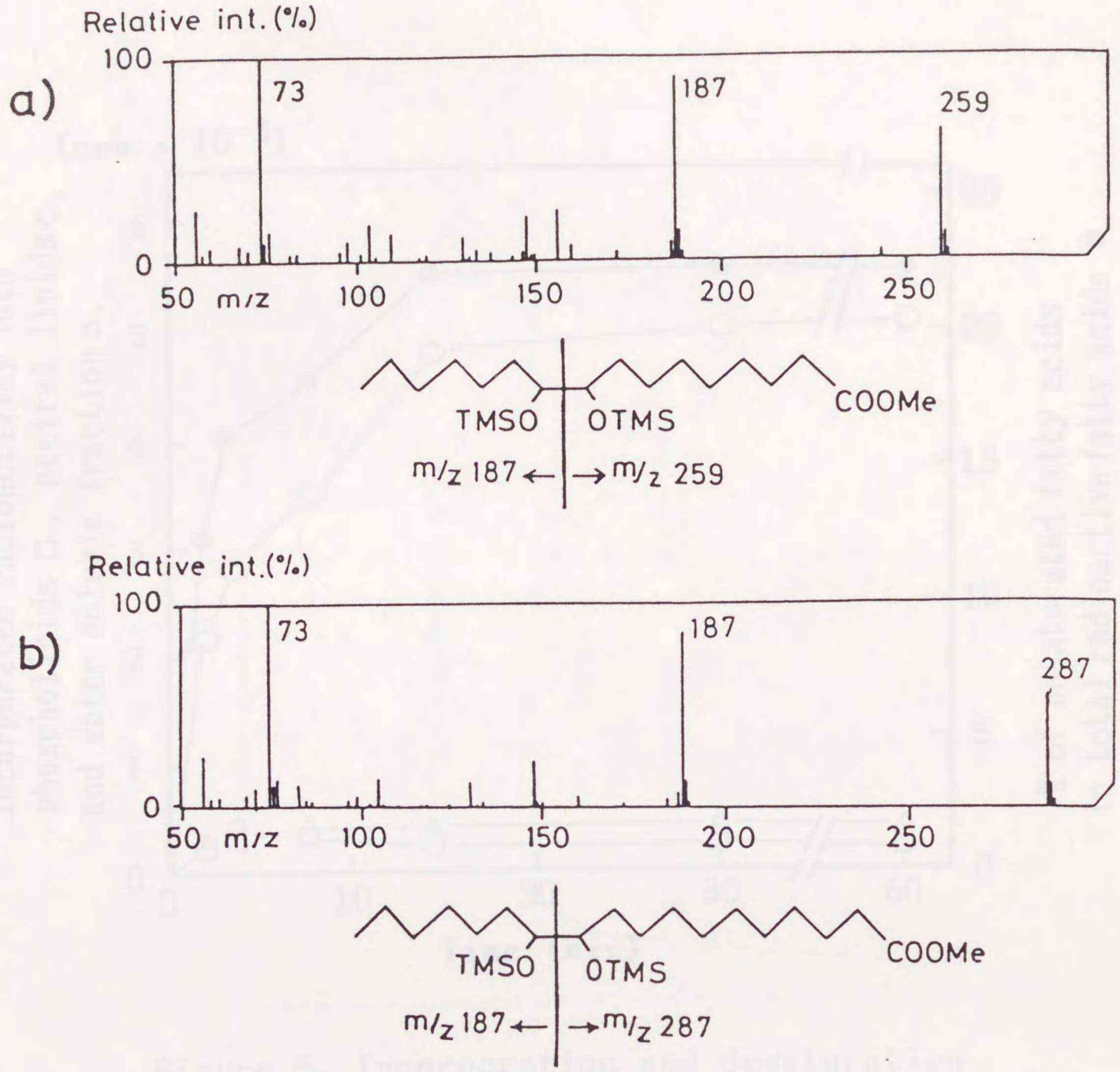


Figure 4. Mass spectra of hexadecenoate (a) and octadecenoate (b) of *Pseudomonas* E-3.

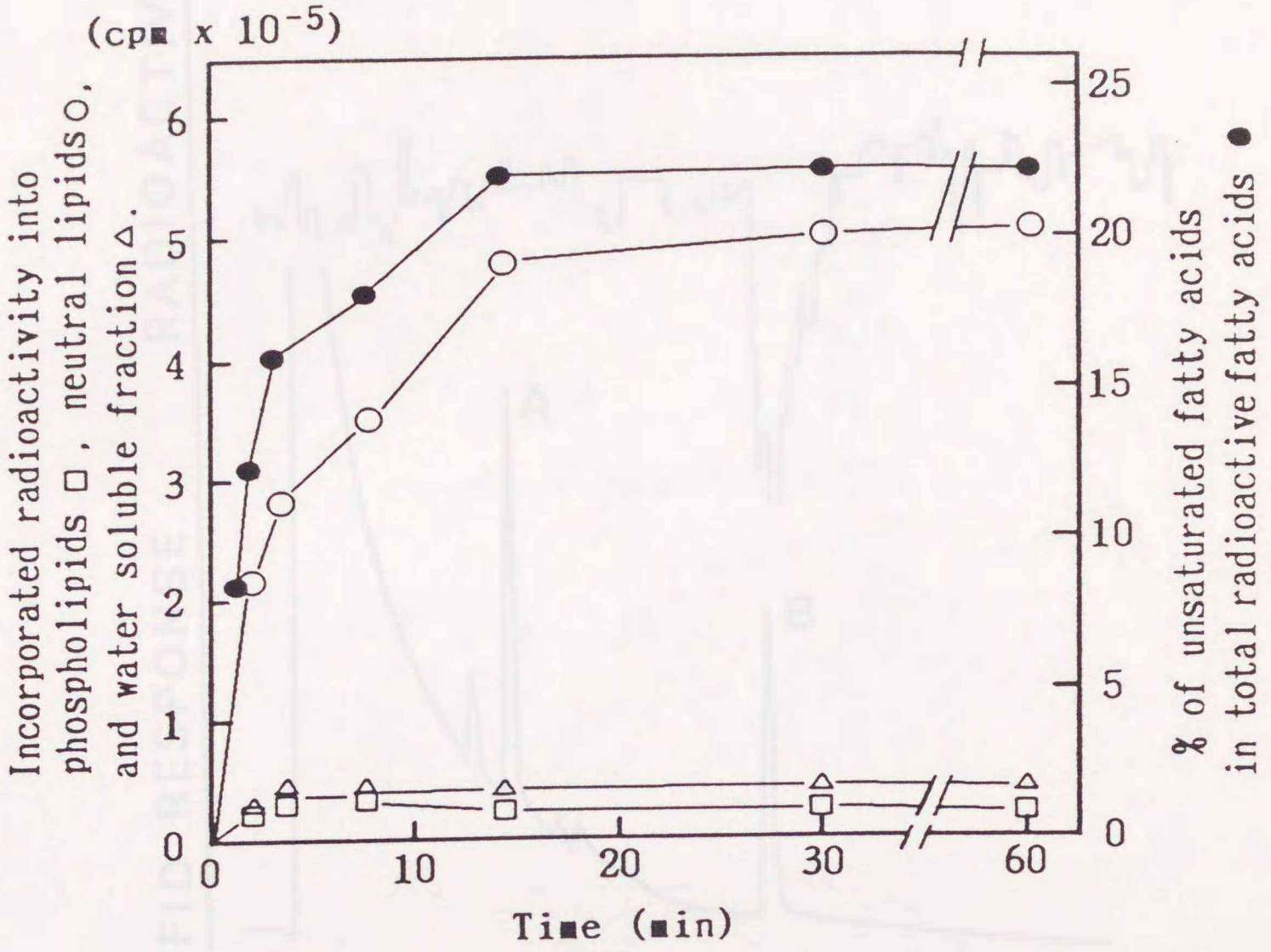


Figure 5. Incorporation and desaturation of [1-^{14}C]pal■itic acid by whole cells

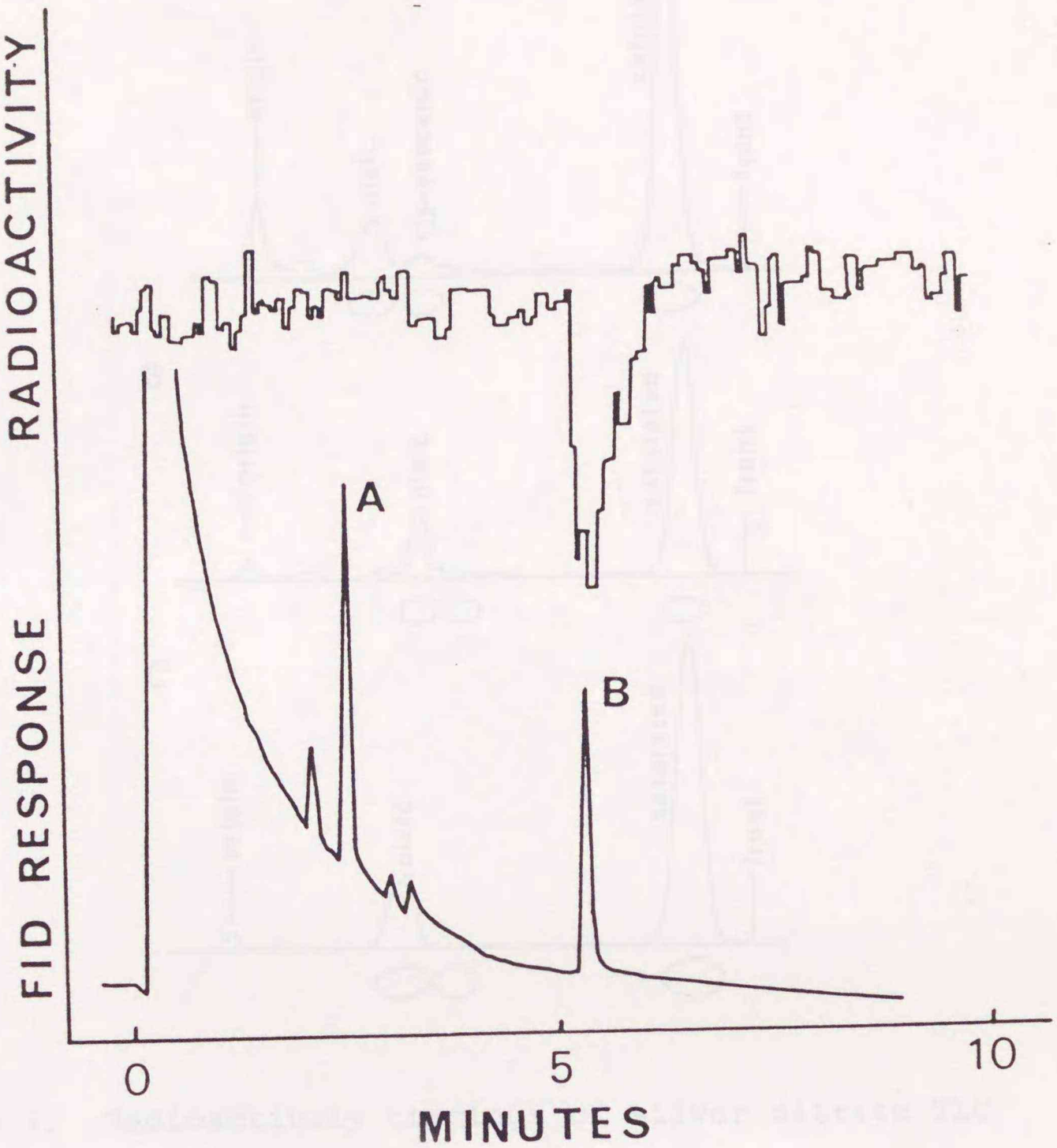


Figure 6. Radio gas chromatogram of methyl esters of oxidation products from [^{14}C]palmitoleate which is produced from [1-^{14}C]lauric acid. Flame ionization detector (FID) response and radioactivity tracing are shown. The methyl esters were separated on a 3 % Silicone SE-30 column (3 mm x 2 m) at N_2 gas flow rate of 30 ml/min. The column temperature was programmed from 100 to 190°C and maintained at 190°C. A, C_7 monocarboxylic acid methyl ester; B, C_{11} dicarboxylic acid methyl ester.

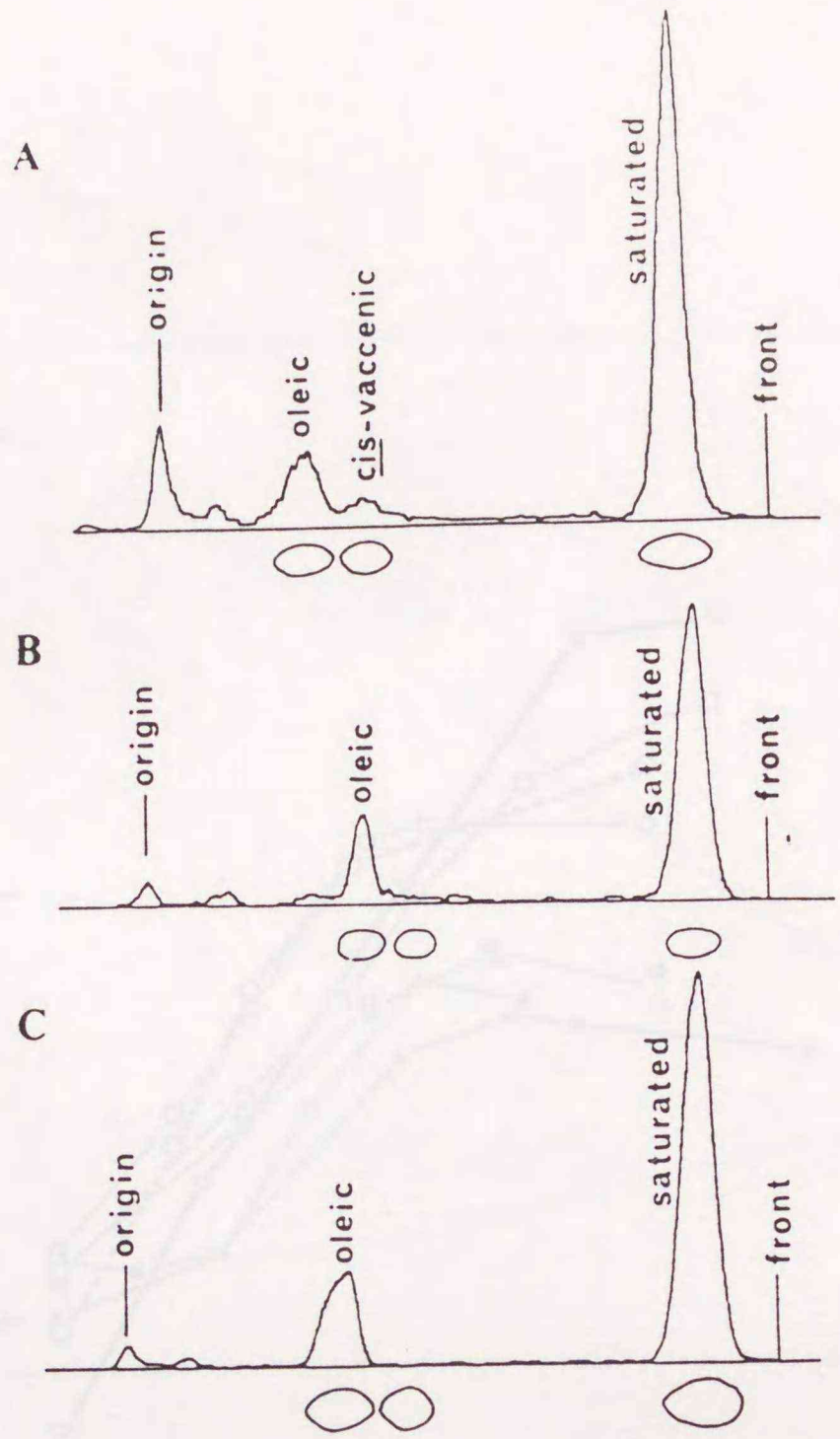


Figure 7. Radioactivity tracings of silver nitrate TLC of fatty acid methyl esters. Fatty acid methyl esters were separated on 20 % silver nitrate impregnated silica plate with toluene as the developing solvent by triple developements at -25°C. Radiolabeled areas were located by a radiochromatogram scanner. A, [1-^{14}C]Stearic acid was added to cell culture and incubated at 15°C for 1 h. B, same as A except that incubation was carried out in the presence of cerulenin (100 µg/ml). C, products of *in vitro* [1-^{14}C]stearyl-CoA desaturase. Reaction was carried out at 25°C for 15 min.

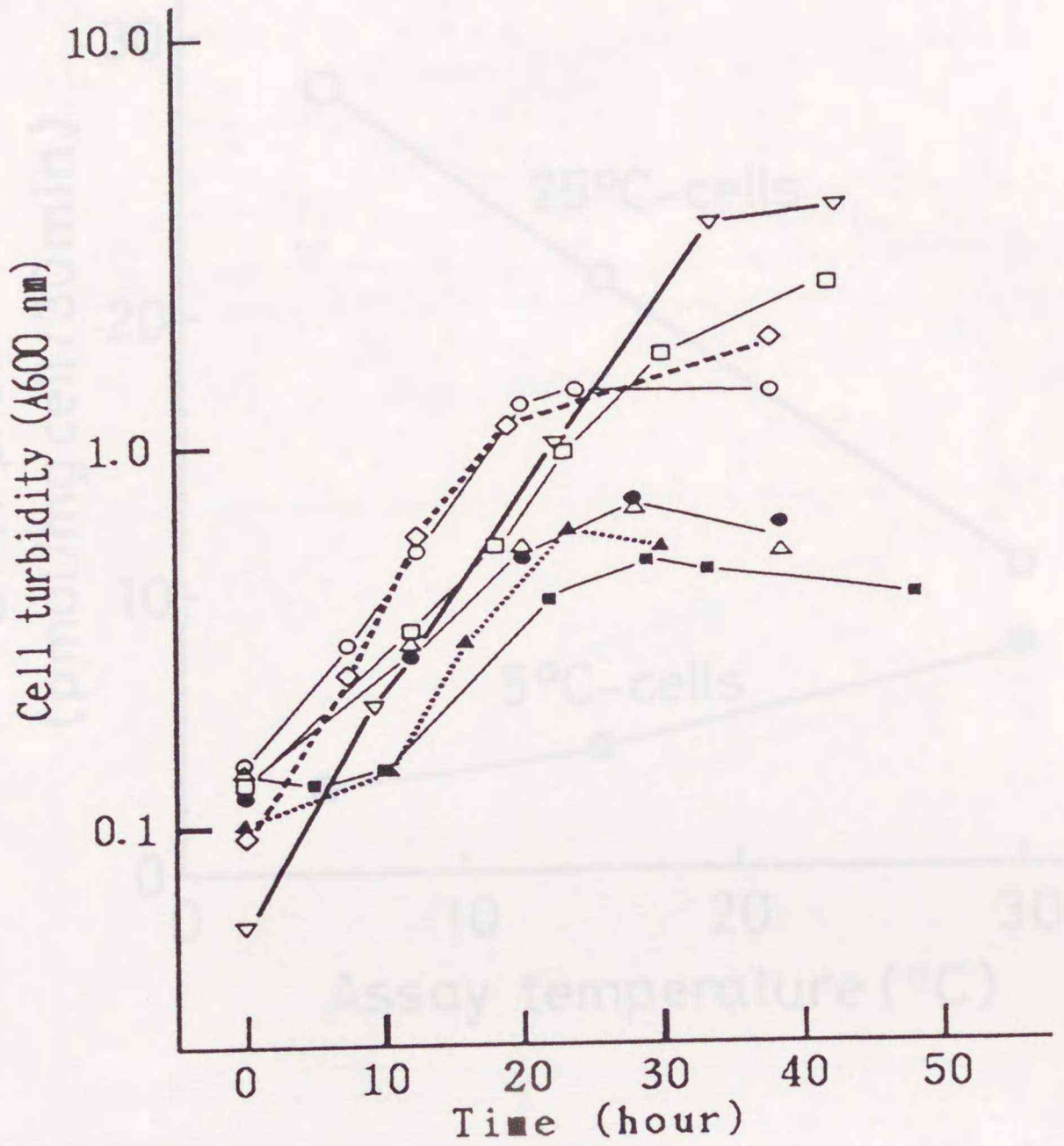


Figure 8. Effect of carbon source on the cell growth at 15°C. ▽, Succinate: ▲, pentadecanoic acid: ●, palmitic acid: □, palmitoleic acid: △, stearic acid: ◇, oleic acid: ○, linoleic acid: ■, hexadecane.

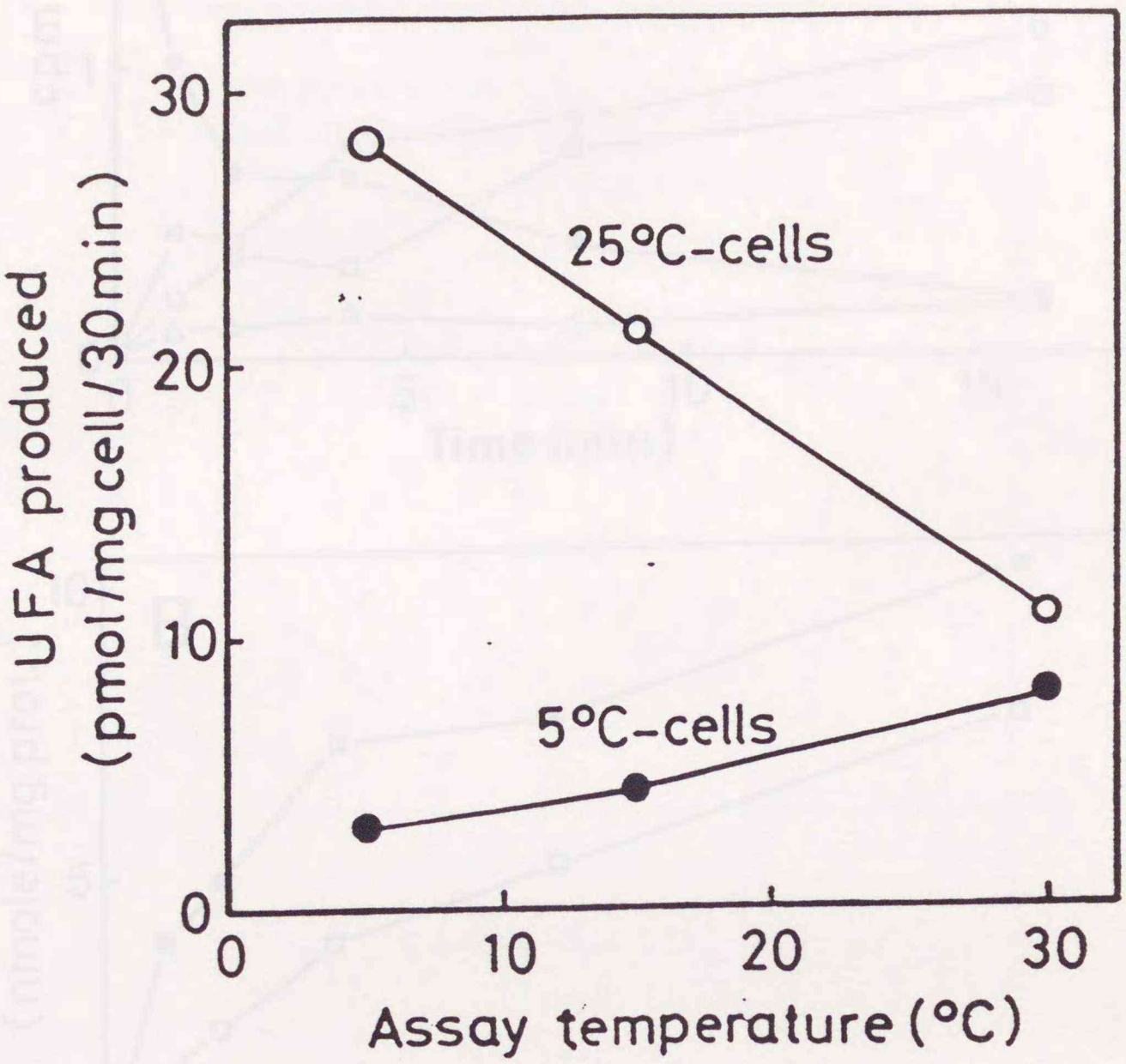


Figure 9. Effects of growth and assay temperatures on the activity of palmitate desaturation by whole cells.

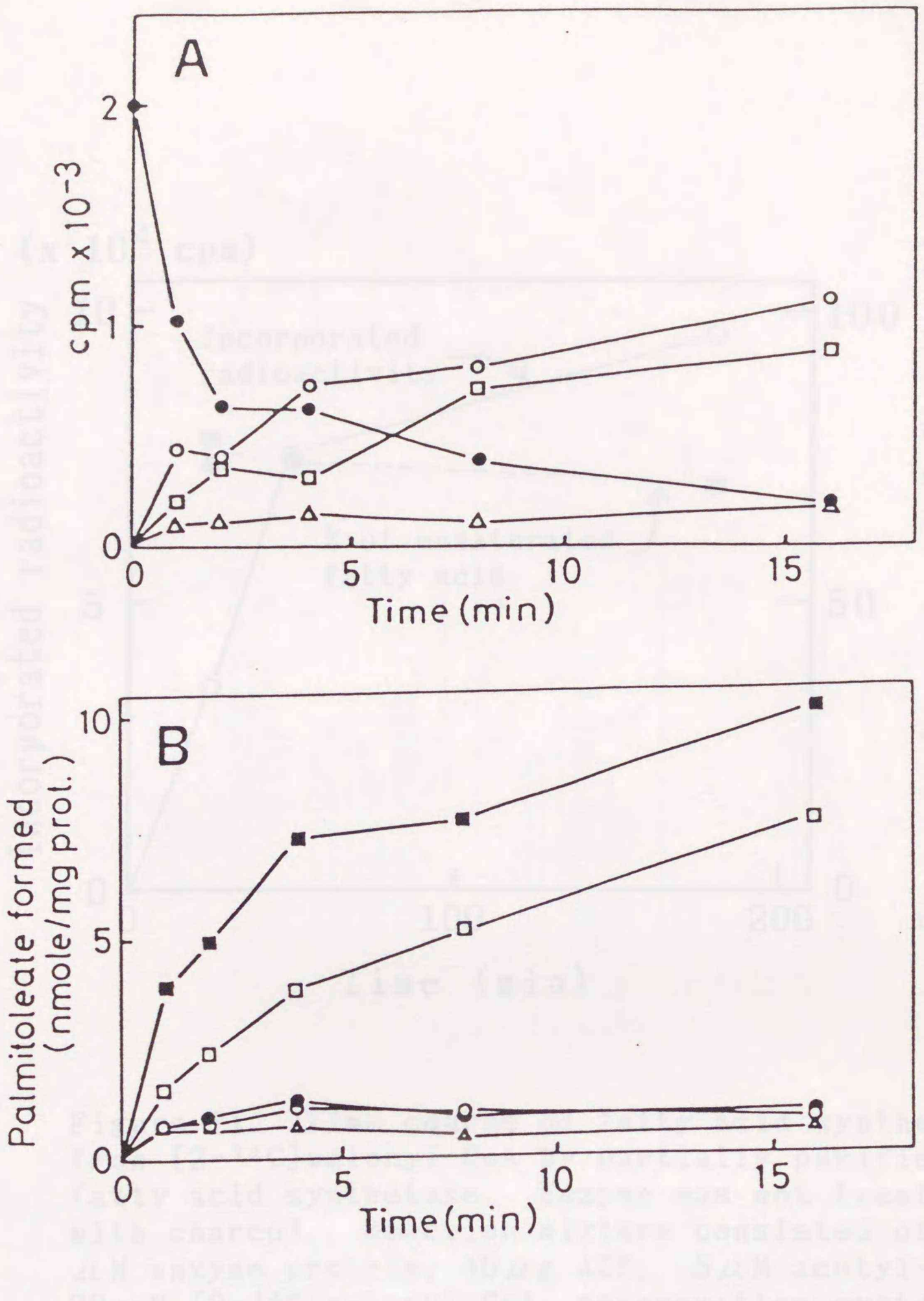


Figure 10. Incorporation and desaturation of [1-^{14}C] palmitoyl-CoA by membrane fraction. A, Icorporation of radioactivity from [1-^{14}C]palmitoyl-CoA to each lipid class. ●, acyl-CoA; ○, phospholipids; □, fatty acids ; △, triacylglycerol. B, Increase of [^{14}C]palmitoleate derived from [1-^{14}C]palmitoyl-CoA in different lipid class. ■, total lipids; □, phospolipids; ●, fatty acids ; ○, triacylglycerol; △, acyl-CoA.

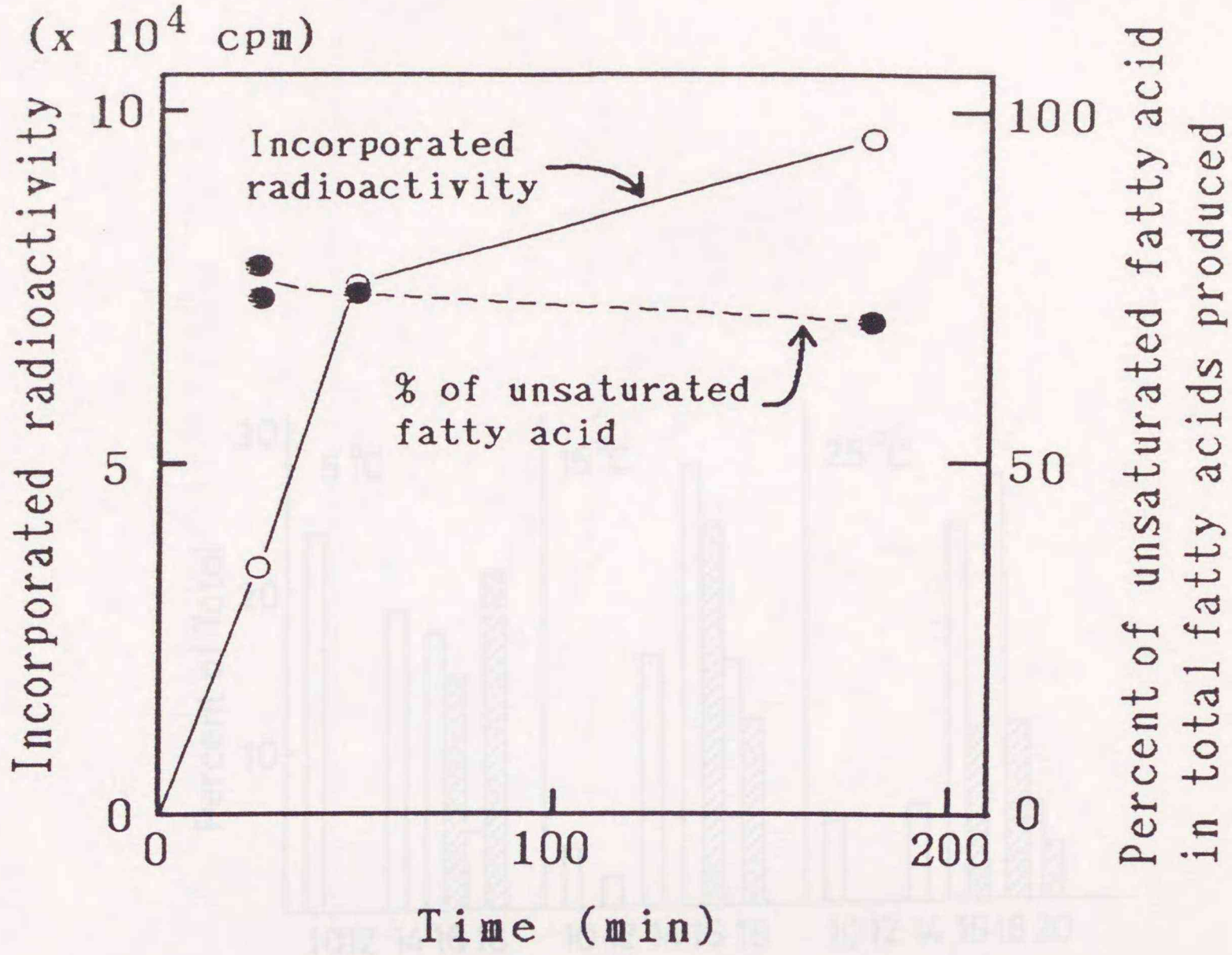


Figure 11. Time course of fatty acid synthesis from [2-^{14}C]malonyl-CoA by partially purified fatty acid synthetase. Enzyme was not treated with charcol. Reaction mixture consisted of 140 μM enzyme protein, 40μg ACP, 5μM acetyl-CoA, 20μM [2-^{14}C]malonyl-CoA, regeneration system of NADPH and NADH in total volume 500μl. Incubation was carried out at 15°C.

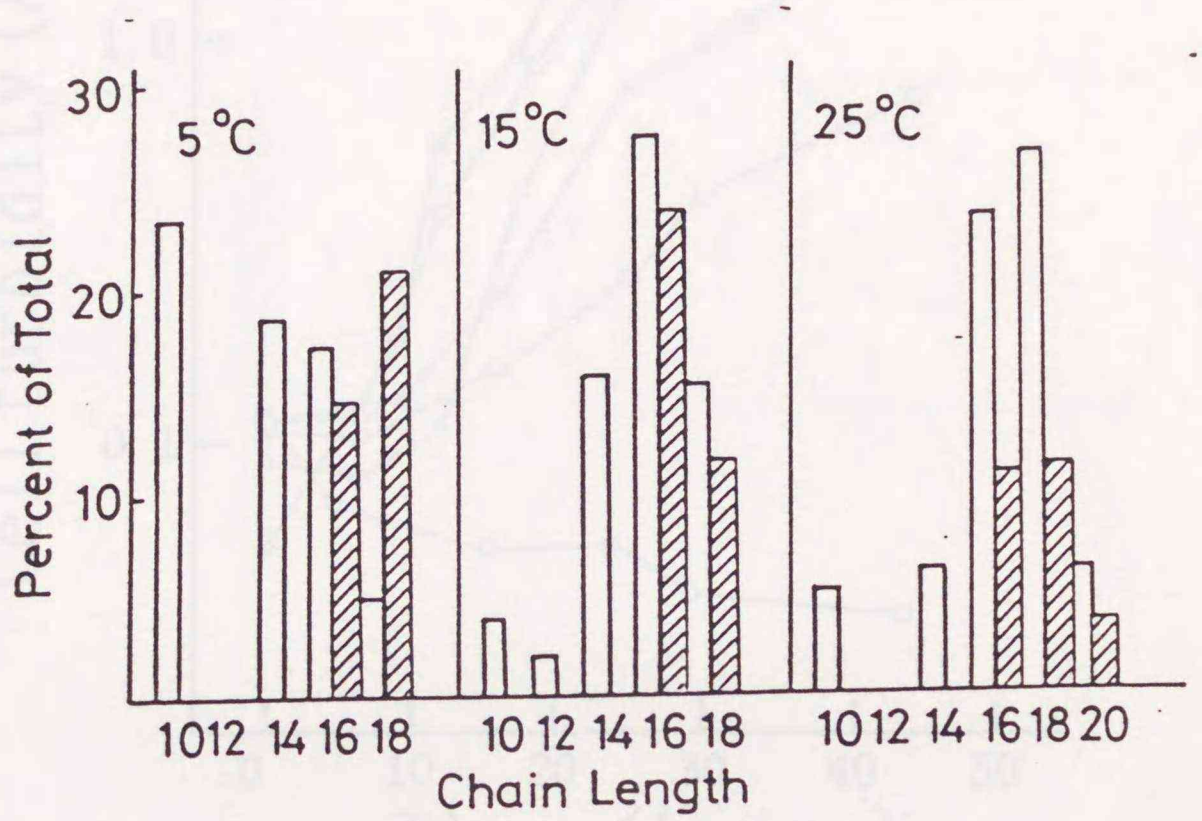


Figure 12. Effect of reaction temperature on the products composition of partially purified fatty acid synthetase. Enzyme was prepared from 15°C-grown cells, and was treated with charcoal. Reaction mixture consisted of 140 μg enzyme protein, 40 μg ACP, 50 μM acetyl-CoA, 20 μM [2-14C]malonyl-CoA, 900 μM NADPH, 900 μM NADH, total volume 500 μl. Incubation was carried out for 1 h. Open and shaded bars indicate saturated and unsaturated fatty acids, respectively.

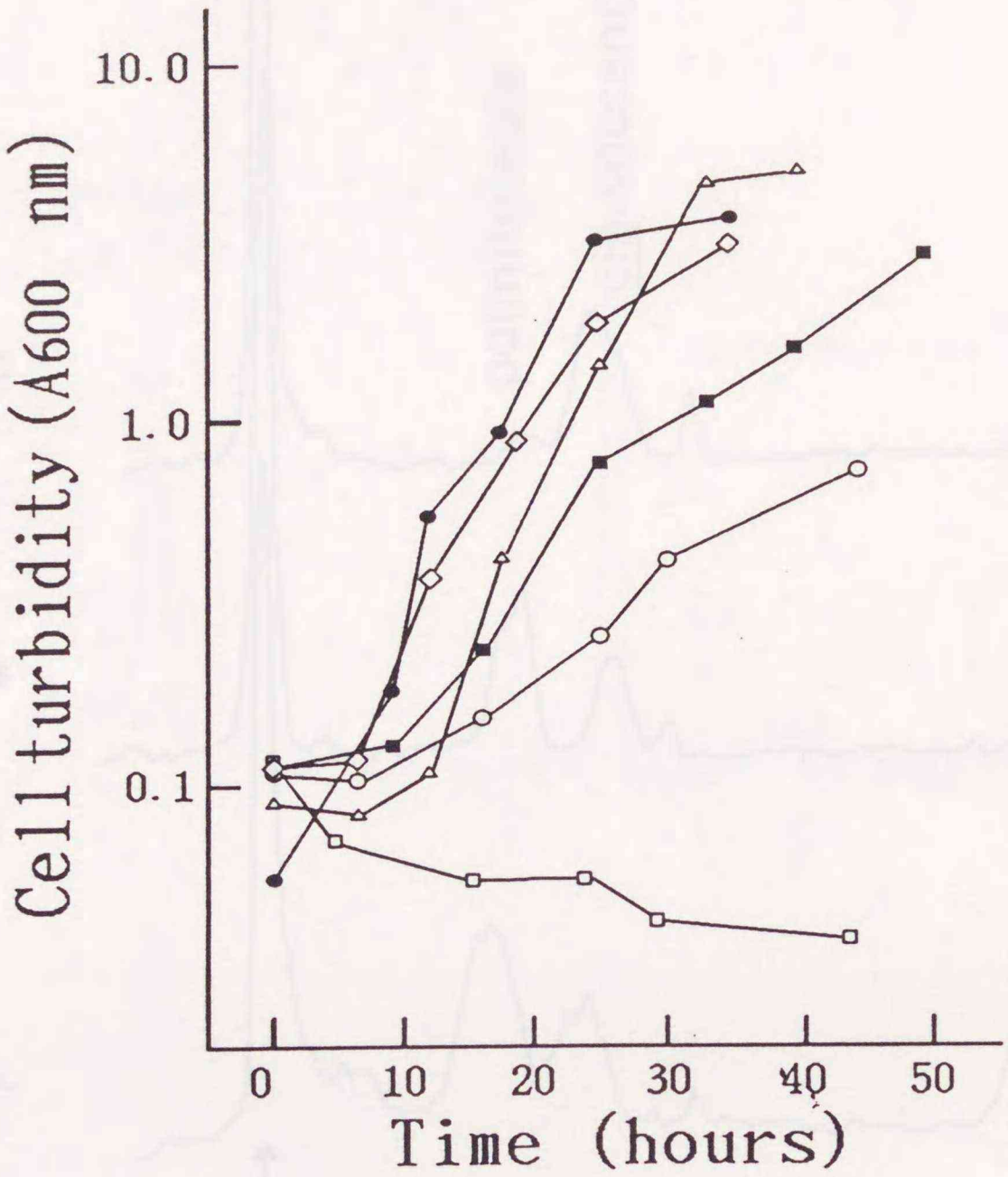


Figure 13. Effect of 3-decynoyl-NAC and supplementation of fatty acid on cell growth. To the culture medium contained succinate (100 mM) and Triton X-100 (0.5 %) was added following concentration of 3-decynoyl-NAC and/or fatty acid. ● , control; ■ , palmitic acid; ◇ , 3-decynoyl-NAC 1 x 10^{-5} M; △ , 3-decynoyl-NAC 1 x 10^{-4} M + oleic acid; ○ , 3-decynoyl-NAC 1 x 10^{-4}M + palmitic acid; □ , 3-decynoyl-NAC 1 x 10^{-4} M.

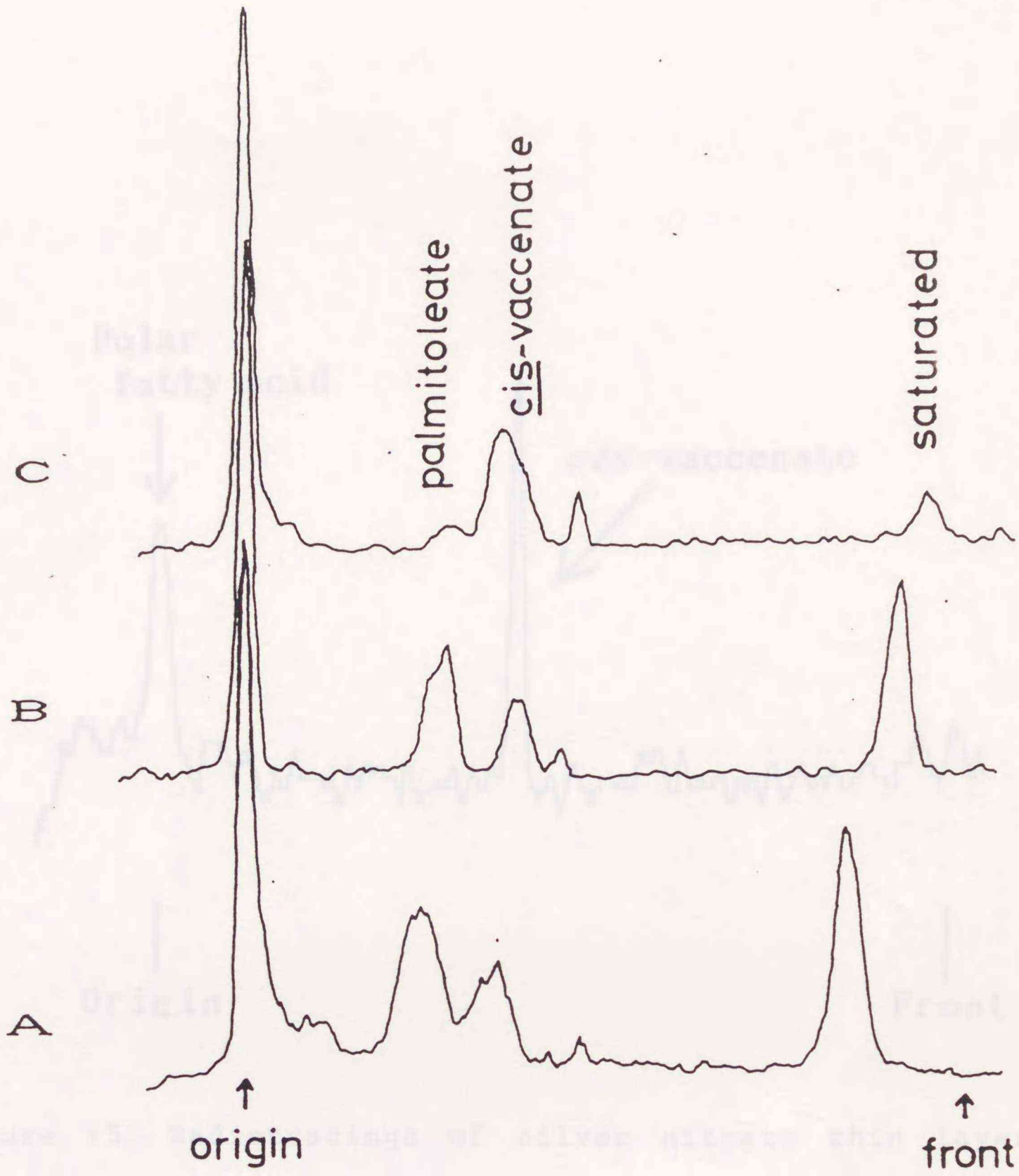


Figure 14. Radiotracings of silver nitrate thin layer chromatograms of fatty acid methyl esters produced in crude cell extract. Samples were developed three times on 20 % silver natrate impregnated silica gel plate with toluene at -25°C. A, Products from [^{14}C]acetate; B, products from [1-^{14}C]acetyl-CoA; C, products from [1-^{14}C]acetyl-CoA and $C_{16:1}$-CoA.

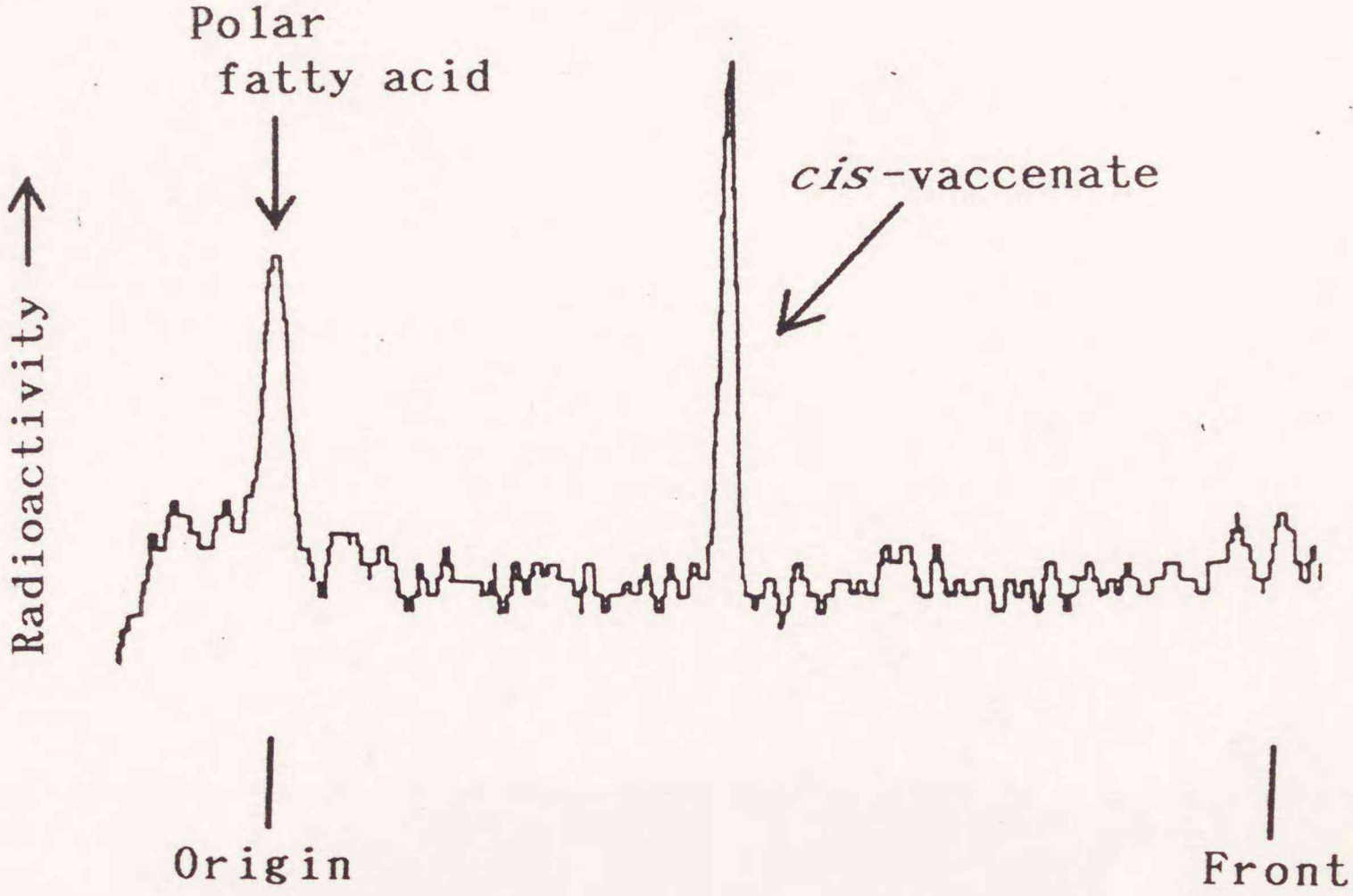


Figure 15. Radiotracings of silver nitrate thin layer chromatogram of fatty acid methyl esters produced in membrane fraction. Reaction mixture containing 50 nmol [1-^{14}C]acetyl-CoA (5 x 10^4 dpm), 50 nmol $C_{16:1}$-CoA, 50 nmol NADPH, 0.5 mg membrane protein, and 0.1 M sodium phosphate buffer in total volume 500 µl was incubated at 15°C for 30 min. Samples were developed on 20 % silver nitrate impregnated silica gel plate with toluene at -25°C.

inches 1 2 3 4 5 6 7 8

cm 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Kodak Color Control Patches

Blue Cyan Green Yellow Red Magenta White 3/Color Black

Kodak Gray Scale

C Y M

A 1 2 3 4 5 6 **M** 8 9 10 11 12 13 14 15 **B** 17 18 19