博　士　論　文

佐藤直樹

昭和 56 年 5 月提出
昭和 56 年 7 月学位授与
東京大学大学院理学系研究科
生物化学専門課程

平成 21 年 1 月　当時の原稿より PDF 化

LIPID BIOSYNTHESIS AND ITS REGULATION BY TEMPERATURE IN *ANABAENA VARIABILIS*

(*Anabaena variabilis* における脂質の生合成とその温度による制御)

NAOKI SATO

佐藤直樹

生物化学専門課程
昭和51年入学
(昭和53年進学)

CONTENTS

CONTENTS (Continued)

Acknowledgements

The author is sincerely grateful to Professor Norio Murata, Department of Biology, University of Tokyo, for valuable discussions and support throughout the work and for reading the manuscript.

He expresses his cordial appreciation to Professors Nobuo Ueta and Yoshiro Miura, Department of Biochemistry, School of Medicine, Teikyo University, for their helpful suggestions in the lipid analysis.

He is indebted to Professor Naotake Morikawa and Miss Teruko Kishikawa, Radioisotope Center, University of Tokyo, for assistance in the operation of radio gas chromatographic apparatus.

The author wishes to thank Professor Yosuke Seyama, Department of Biochemistry, Faculty of Medicine, University of Tokyo, for his guidance and assistance in gas chromatography-mass spectrometry measurements.

Abbreviations

Lipids

GlcDG, monoglucosyl diacylglycerol

GalDG, monogalactosyl diacylglycerol

Gal_2DG, digalactosyl diacylglycerol

Gal_3DG, trigalactosyl diacylglycerol

SqDG, sulfoquinovosyl diacylglycerol

PG, phosphatidylglycerol

PC, phosphatidylcholine

PA, phosphatidic acid

PE, phosphatidylethanolamine

PI, phosphatidylinositol

PS, phosphatidylserine

DPG, diphosphatidylglycerol

DG, diacylglycerol

Fatty acids

Fatty acids are expressed by numbers of carbon atoms and of double bonds. Positions of double bonds are indicated, if necessary, in parenthesis. In Chapters 2 to 6, fatty acids of Anabaena variabilis are abbreviated as follows:

16:0, palmitic acid

16:1, palmitoleic acid (9)

16:2, hexadecadienoic acid (double bond positions are not known)

18:0, stearic acid

18:1, oleic acid (9)

18:2, linoleic acid (9,12)

18:3, linolenic acid (9,12,15)

Lipid molecular species

Lipid molecular species are expressed by a combination of abbreviated forms of fatty acids, e.g.,

18:1/16:0, sn-1-oleoyl-2-palmitoyl species

Others

ACP, acyl carrier protein

CoA, coenzyme A

TLC, thin-layer chromatography

GLC, gas-liquid chromatography

T_f, temperature for the onset of phase separation of membrane lipids (cf. Chapter 1, I. (4) (a))

Gly, glycerol (in Chapter 5)

TMS, trimethylsilyl

Chapter 1

Introduction

Contents

I. Blue-green algae ——— a comparison with the chloroplasts of eukaryotic organisms

(1) Cellular architecture, taxonomy and growth properties

Blue-green algae [1,2,3] (Myxophyceae , Cyanophyceae[1], or cyanobacteria [4]) are prokaryotes that perform oxygenic photosynthesis [2-4]. The blue-green algal cell is confined by a cell envelope which consists of outer membrane, peptidoglycan and cytoplasmic (inner) membrane[4]. It possesses, in the cytoplasm, several layers of thylakoid membrane which is the site of photosynthetic primary reactions.

The blue-green algae are classified into five orders [1], i.e., Chroococcales [5], Chamaesiphonales, Pleurocapsales [6], Nostocales and Stigonematales. Anabaena strains are filamentous and belong to Nostocales. Although most strains of Anabaena can form heterocysts in which molecular nitrogen is fixed [3,4], a strain M-3 of Anabaena variabilis (from the Algal Collection of the Institute of Applied Microbiology, University of Tokyo) used in the present study does not form the heterocysts and does not fix molecular nitrogen. Anabaena variabilis strain M-3 can grow normally at temperatures ranging from 20 to 38°C [7,8].

(2) Lipid components

(a) Lipid classes

The blue-green algae contain four major lipid classes, i.e.,monogalactosyl diacylglycerol (GalDG), digalactosyl diacylglycerol (Gal_2DG), sulfoquinovosyl diacylglycerol (SqDG) and phosphatidylglycerol (PG) (Fig. 1) [2-4, 8-10], and, in addition, some other glycolipids as minor

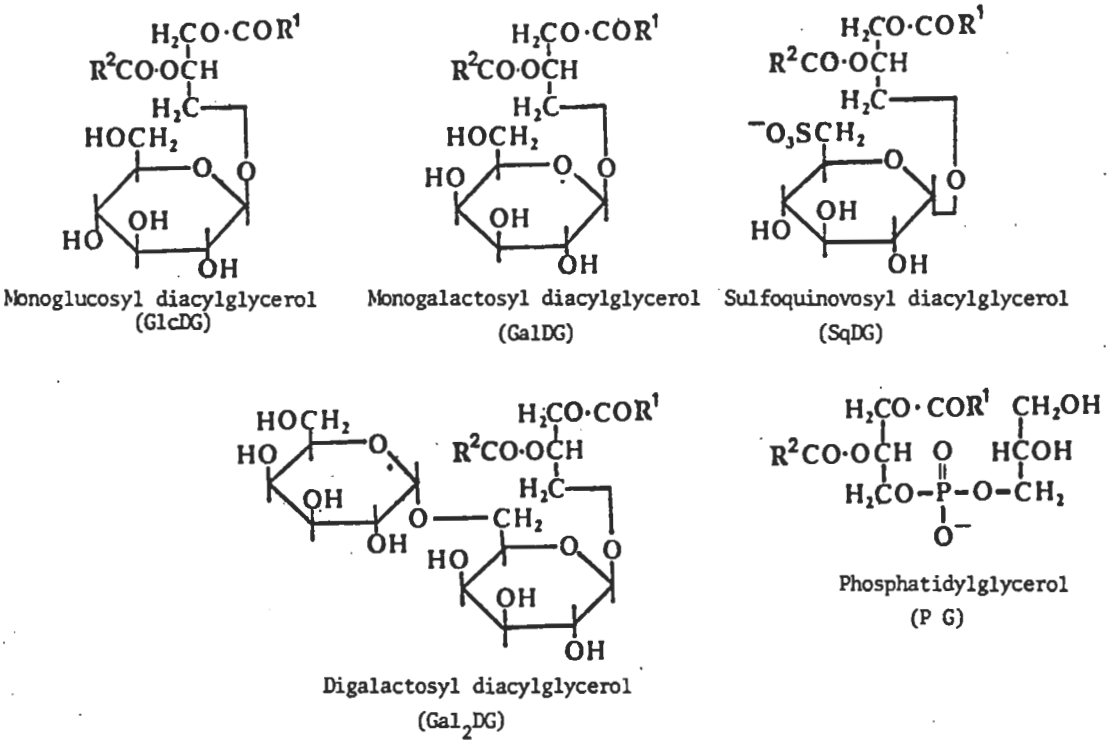

Fig. 1 Lipids present in blue-green algae.

components, i.e., monoglucosyl diacylglycerol (GlcDG) [11] and trigalactosyl diacylglycerol (Gal_3DG) [12]. They do not contain phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), diphosphatidylglycerol (DPG) and phosphatidylserine (PS) [8-10,15]. Chloroplasts of higher plants contain GalDG, Gal_2DG, SqDG and PG as the major components and PC, PI and Gal_3DG as minor components [13,14].

(b) Fatty acid composition

The blue-green algae are classified into four groups in respect to fatty acid compositions [10, 12, 16-20]: Strains in the first group contain little or no polyunsaturated fatty acids; those in the second group contain 18:2 and 18:3(9,12,15); those in the third group contain 18:2 and 18:3(6,9,12); those in the fourth group contain 18:4 in addition to 18:2, 18:3(9,12,15) and/or 18:3(6,9,12). Unicellular strains of the blue-green algae (Chroococcales) belong to the first and third groups [5,17,21]. Filamentous blue-green algae (Nostocales) all contain polyunsaturated acids and belong to the second, third and fourth groups. Anabaena variabilis M-3, which contains 16:0, 16:1(9), 16:2, 18:0, 18:1(9), 18:2(9,12) and 18:3(9,12,15), belongs to the second group [8]. Since the chloroplasts of most higher plants contain 18:2(9,12) and 18:3(9,12,15) as the major fatty acids, they correspond to the second group of blue-green algae. The chloroplasts of some other plants contain 18:4 in addition, and correspond to the fourth group [22].

In the blue-green algae, the fatty acid compositions of individual lipid classes are similar to one another. However, contents of the monounsaturated fatty acids relative to those of the saturated ones are higher in galactolipids (GalDG and Gal_2DG) than in SqDG and PG in Anacystis nidulans [8,9] and Synechococcus lividus [23] (the first group). The

galactolipids (GalDG, Gal_2DG and Gal_3DG, if present) contain greater amounts of polyunsaturated fatty acids (18:3 or 18:4) than SqDG and PG in Anabaena variabilis [8,9], Anabaena cylindrica [12], Nostoc calcicola[12] Oscillatoria chalybea [12] (the second group), Spirulina platensis [10] (the third group) and Tolypothrix tenuis [12](the fourth group).

In the chloroplasts of higher plants, the fatty acid compositions are different among the lipid classes [13, 14]. More than 95 % of the fatty acids in GalDG are 18:3(9,12,15) and 16:3(7,10,13). The major fatty acids in Gal_2DG are 16:0 and 18:3. SqDG contains 18:3 and 16:0, while PG contains 18:3, 16:1(3, trans) and 16:0. PC and PI contain 16:0, 18:1, 18:2 and 18:3.

(c) Positional distribution

Positional distributions of the fatty acids at the C-1 and C-2 positions of sn-glycerol moiety are determined by selective enzymatic hydrolysis of the ester linkage at the C-1 position [24]. Two types of the positional distributions are known. In case of Anacystis nidulans, the monounsaturated fatty acids (14:1, 16:1 and 18:1(9 and 11)) are esterified to the C-1 position and 16:0 is to the C-2 position in all the lipid classes [8]. In the filamentous blue-green algae, Anabaena [8,12,25], Oscillatoria [12], Nostoc [12] and Tolypothrix [12], the C_{18} acids are esterified to the C-1 position, and the C_{16} acids are to the C-2 position in all the lipid classes. In the chloroplasts of higher plants[26,27] and green algae [25], the positional distributions of fatty acids in GalDG, SqDG and PG are similar to those in the filamentous blue-green algae, although they are not so strictly selective as in the blue-green algae.

(d) Lipid molecular species

Lipid molecular species is characterized by a combination of the acyl groups at the C-1 and C-2 positions as well as the polar substituent group at the C-3 position. Compositions of the lipid molecular species are known in a limited number of blue-green algal strains in which the fatty acids are esterified to the C-1 and C-2 positions according to the number of carbon atoms [12,28].

The most abundant molecular species in all the lipid classes are 18:3/16:0 in Anabaena cylindrica, Oscillatoria chalybea and Nostoc calcicola and 18:4/16:0 in Tolypothrix tenuis [12]. In Anabaena variabilis M-3 [28], the major molecular species in cells grown at 38°C are 18:1/16:0 and 18:2/16:0 in SqDG and PG, and 18:1/16:0, 18:2/16:0, 18:1/16:1 and 18:2/16:1 in GalDG and Gal_2DG. The major species in cells grown at 22°C are 18:2/16:0 and 18:3/16:0 in SqDG and PG, and 18:2/16:0, 18:3/16:0, 18:3/16:1 and 18:3/16:2 in GalDG and Gal_2DG.

In the chloroplasts of higher plants [29], the major molecular species are 18:3/18:3 and 18:3/16:3 in GalDG, while they are 18:3/18:3 and 16:0/18:3 in Gal_2DG. The most abundant molecular species in SqDG and PG are 18:3/16:0 and 18:3/16:1(3, trans), respectively.

(3) Previous studies on the lipid metabolism

(a) Blue-green algae

Nichols et al. [9] first studied the lipid metabolism in the blue-green algae, Anacystis nidulans and Anabaena variabilis. They found that when the cells were incubated with ^{14}C-acetate, the label was incorporated into GalDG, Gal_2DG, SqDG and PG. They also found that exogenously added radioactive 16:0, 18:0 and 18:1 were desaturated to 16:1, 18:1 and 18:2,

and 18:2 and 18:3, respectively, in Anabaena variabilis.

Nichols[30], using Anacystis nidulans and Anabaena cylindrica, found that the radioactivity shifted from the saturated to the unsaturated fatty acids in GalDG and SqDG. A shift of the radioactivity from C_{14} to C_{16} acids was also observed in Anacystis nidulans. He pointed out that the complex lipids that were structural components of the photosynthetic membranes served as intermediates of the fatty acid synthesis. Appleby et al.[15], on the basis of similar findings, inferred that GalDG might be involved in the desaturations of fatty acids in Anabaena variabilis.

Feige [31] discovered, in a wide variety of blue-green algae, a new lipid component, which had a slightly higher mobility in TLC than GalDG did. The content of the lipid was less than 0.1 % of the total lipids. Feige et al.[11] tentatively proposed a structure, β-glucosyl diacylglycerol (GlcDG), for the lipid, though they could not definitely determine the chemical structure. A study based on pulse-labeling and chase experiment led them to consider that GlcDG might be a precursor of GalDG.

In spite of these findings, a general view of the lipid metabolism in the blue-green algae has not been established.

(b) Photosynthetic eukaryotes

In the chloroplasts of higher plants and eukaryotic algae there exist large amounts of galactolipids that contain polyunsaturated fatty acids [22, 32-35]. Biosyntheses of the galactolipids and the polyunsaturated fatty acids have been studied in Chlorella, Euglena and higher plant leaves [36-41].

(i) Chlorella vulgaris

In Chlorella vulgaris, the saturated fatty acids are synthesized

at first, and then desaturated to the mono- and polyunsaturated fatty acids after they are esterified to the complex lipids [42,43]. The direct desaturation of 18:1 in PC was first demonstrated in this alga [44-46, 16]. The desaturations of 18:1 to 18:2, and of 16:0 to 16:1 and to 16:2, while they are bound to GalDG, have also been postulated[26, 47].

(ii) Euglena gracilis

A desaturase system which converts 18:0-ACP to 18:1-ACP is well characterized in Euglena gracilis [48-50]. It is composed of NADPH-oxidase, ferredoxin and a terminal desaturase. The desaturation reaction requires molecular oxygen and NADPH as cofactors. The terminal desaturase is a soluble non-heme iron protein.

(iii) Higher plants

A scheme for the lipid biosynthesis in higher plant leaves proposed by Roughan et al.[51] is presented in Fig. 2. There are two possible pathways for the production of GalDG. The fatty acid synthetase (dissociable type) which is localized in the chloroplast produces 16:0-ACP [52]. Palmitoyl-ACP is elongated to 18:0-ACP [53], which is subsequently converted to 18:1-ACP [54]. Then, in the first pathway, ACP is replaced by CoA. Oleoyl-CoA is transported to the endoplasmic reticulum and 18:1 is incorporated to PC[51,55-57]. Dioleoyl-PC is converted to 18:2/18:2-PC by desaturation of 18:1[55, 56]. Then, the phosphocholine group is removed from PC, and the resultant DG is transported back to the chloroplast envelope. There, a galactose unit is transferred to dilinoleoylglycerol to form 18:2/18:2 species of GalDG[58-60], which is then converted to 18:3/18:3 species by desaturation of 18:2 [55, 56, 60].

In the second pathway, DG is produced from 18:1 and 16:0 within the

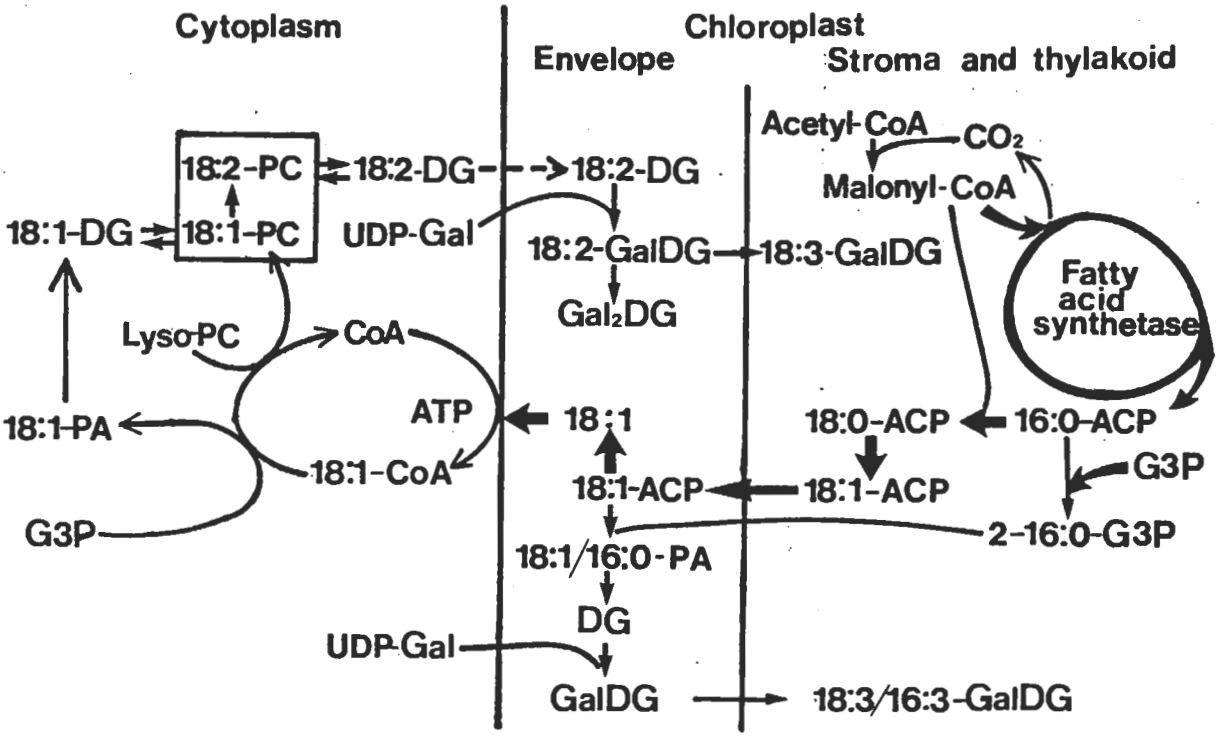

Fig. 2 A scheme for the lipid biosynthesis in higher plants according to Roughan et al. [51]. Bold arrows indicate the predominant pathway of fatty acid metabolism in isolated chloroplasts. G3P, glycerol-3-phosphate; UDP, uridine diphosphate.

chloroplast [51]. The fatty acids in GalDG are then desaturated to 18:3 and 16:3 [61-63]. In most of the higher plants, GalDG is produced by the first pathway. In some plant species (such as spinach), however, both pathways seem to operate. According to Siebertz et al. [63], most of GalDG is synthesized by the second pathway in spinach, whereas Roughan et al.[51] infer that the contribution of the second pathway is only 20 %.

The direct desaturation of 18:1 in PC in the endoplasmic reticulum [64, 65] is demonstrated by tracer experiments in vitro. The direct desaturation of 18:2 to 18:3 in PC is also observed in soya bean cotyledons [66]. The desaturation of fatty acids in GalDG is not characterized yet.

Digalactosyl diacylglycerol (Gal_2DG) is synthesized in the chloroplast envelope [59, 67]. Two mechanisms are proposed for the biosynthesis of Gal_2DG. In one mechanism, a galactose unit is transferred from UDP-galactose to GalDG [68, 69]. In the other mechanism, a galactose unit is transferred from one GalDG molecule to another (dismutation) to form one Gal_2DG and one DG molecules [70]. The fact that Gal_2DG contains a greater amount of 16:0/18:3 molecular species than GalDG does [29] suggests that the molecular species composition of the precursor GalDG is different from that of bulk GalDG [60 , 63]. The mechanisms for the biosyntheses of SqDG and PG are not established.

(4) Temperature effects

(a) Physiological effects

An organism is alive in a limited temperature range. Above the upper limit, enzymes are inactivated by denaturation [71, 72], whereas, below the lower limit, lipids in the biological membranes solidify [72-79].

In most cases, the bilayer lipids of membranes are in the liquid-crystalline state under the growth conditions. When the temperature is lowered, the fluidity of membrane continuously decreases. When the temperature reaches a critical value (T_f), domains of the gel state appear in the membrane. With decrease of temperature below T_f, the domains of gel state expand and finally the whole membrane becomes in the gel state [74, 80-83]. A state in which the domains of gel and liquid-crystalline states coexist is called the phase separation state. When the membrane is in the gel state, the motional freedom of enzymes and lipid molecules is reduced and thus the membrane-bound enzymes lose their activities[84-88]. The membrane in the phase separation state loses its function as a permeability barrier to small molecules[74,89-91]. The maintenance of membrane fluidity at low temperatures is, therefore, crucial for survival of the microorganisms and the plants [35,72].

A typical case of the thermo-adaptive change in the membrane fluidity is seen in Anacystis nidulans in which the temperature for the onset of phase separation shifts in parallel with growth temperature. T_f of the thylakoid membrane is about 25 and 15°C in cells grown at 38 and 28°C, respectively [82,83,92,93]. The temperatures for the maximum yield of chlorophyll a fluorescence [92,94], and for the discontinuity in the Arrhenius plots of the photosynthetic oxygen evolution [92], the state 1-state 2 shift [92] and the photophosphorylation [95] are equal or very close to T_f. The yield of the delayed fluorescence of chlorophyll a changes from a high to low level at T_f [7].

T_f's detected by the temperature-dependence of delayed fluorescence appear at 13 and 8°C in Anabaena variabilis strain M-3 grown at 38 and 20°C, respectively [7]. Characteristic break points in the

Arrhenius plots of the cytochrome reduction and the state 1- state 2 shift also vary with growth temperature in Synechococcus lividus [23].

(b) Temperature-dependent changes in fatty acids of blue-green algae

(i) Variation with temperature in isothermal growth at different temperatures

Holton et al.[21] compared the fatty acid compositions of the total lipids from cells of Anacystis nidulans which were isothermally grown at 26, 32, 35 and 41°C. They found that the ratio of 16:1 to 16:0 was higher and the average number of carbon atoms in fatty acids was larger at lower growth temperatures. Sato et al.[8] and Murata et al.[93] observed similar changes in the fatty acid compositions of all the lipid classes in Anacystis nidulans grown at 22, 28 and 38°C. They also demonstrated that with decrease of the growth temperature, the average chain length of monounsaturated acids was reduced at the C-1 positions of all the lipid classes, and 16:1 increased and 16:0 concomitantly decreased at the C-2 positions of GalDG and Gal_2DG.

When the growth temperature was decreased in a thermophilic unicellular blue-green alga, Synechococcus lividus [23], 16:1 and 18:1 increased and 18:0 decreased in PG and SqDG, and 16:1 increased and 16:0 and 18:0 decreased in GalDG and Gal_2DG.

In contrast to these chroococcacean blue-green algae, Anabaena variabilis contains the polyunsaturated fatty acids and responds to the growth temperature in a different way; with decrease of the growth temperature, 18:1 and 18:2 decrease and 18:3 increases at the C-1 positions of all the lipid classes, while 16:0 decreases and 16:2 increases at the C-2 positions of GalDG and Gal_2DG[8, 96].

The increase in the unsaturation and the decrease in the chain length of fatty acids are known to lower the temperature of phase transition of membrane lipids [74-76]. Therefore, the changes in fatty acid compositions with growth temperature can be regarded as thermo-adaptive responses in these algae [7, 23, 92, 93].

(ii) Changes after temperature shift

Apart from the temperature-dependent variations in fatty acid compositions under the isothermal growth conditions, a rapid and transient modification of fatty acids occurs after an abrupt shift of temperature in Anabaena variabilis [28, 96]. For 10 h after the temperature shift from 38 to 22°C, a decrease in 16:0 and a concomitant increase in 16:1 take place at the C-2 position of GalDG. These rapid changes are canceled afterwards by reverse changes. Decreases in 18:1 and 18:2 and an increase in 18:3 in GalDG, SqDG and PG are slower than the changes in the C_{16} acids in GalDG. As to the molecular species composition [28], 18:1/16:0 molecular species of GalDG decreases for 10 h after the temperature shift and those of SqDG and PG do more slowly. Sato and Murata [28, 96] have proposed that the rapid and transient decrease in 16:0 and increase in 16:1 at the C-2 position of GalDG play a central role in the temperature acclimation in Anabaena variabilis.

A downward temperature shift from 38 to 28°C provokes rapid and slow changes in the fatty acid compositions in Anacystis nidulans [93]. Almost all of 16:0, which is bound to the C-1 positions of GalDG, SqDG and PG at 38°C, is rapidly converted to 16:1 within 10 h after the temperature shift. Decreases in chain length of the monounsaturated fatty acids at the C-1 positions of all the lipid classes and desaturation of 16:0 to 16:1 at the C-2 positions of galactolipids proceed only

slowly. It is reasonable to assume that the rapid desaturation of 16:0 to 16:1 at the C-1 positions of the lipids plays an important role in the temperature acclimation in Anacystis nidulans.

(c) Temperature-dependent changes in fatty acids of photosynthetic eukaryotes

Cyanidium caldarium can grow in a temperature range between 20 and 55°C [97]. Under both of the photoautotrophic [98] and heterotrophic growth conditions [97], 18:3 increases and 16:0 decreases with decrease of temperature. Upon a temperature shift from 53 to 25°C, the cells begin to synthesize 18:3 after an induction period of 48 h[99].

In Chlorella sorokiniana grown under isothermal conditions [100], the proportion of unsaturated fatty acids increases and the average chain length of fatty acids decreases with decrease of temperature.

During the greening of Euglena gracilis under illumination, a greater amount of 18:3 is synthesized and esterified to GalDG and Gal_2DG at 20°C than at 28°C [101].

In the leaves of Vicia faba grown isothermally between 5 and 30°C [102], an increase in 18:3 and a concomitant decrease in 18:2, 18:1 and 16:0 are seen with decrease of temperature. Upon a temperature shift from 30 to 20°C, 18:3 in GalDG gradually increases to the level at 20°C in 2 days.

Increases in 18:3 occur also in PC of linseed and soya bean cotyledons in 2 days after a downward temperature shift [103]. A similar change in 18:3 is observed in phospholipids of soya bean root plasma and mitochondrial membranes [104]. The mechanism of temperature-dependent changes in the contents of 18:3 is not known at present.

II. Lipid metabolism and its regulation by temperature in non-photosynthetic microorganisms

(1) Bacteria

(a) Escherichia coli

Lipid biosynthesis and the effect of temperature on it are most extensively studied in this bacterium. PE, PG and DPG are major lipid components, and palmitic (16:0), palmitoleic (16:1(9,cis)), cis-vaccenic (18:1(11, cis)) and cyclopropane acids (cyclo 17:0 and cyclo 19:0) are major fatty acids [105]. 16:0, 16:1 and 18:1 are synthesized from acetyl CoA and malonyl CoA by fatty acid synthetases of a dissociable type [87, 106-108]. The synthesis of the unsaturated fatty acids does not require oxygen and is termed "anaerobic pathway": The double bond is introduced during the fatty acid synthesis by 3,4-dehydration of β-hydroxydecanoyl-ACP, and the resultant 10:1(3, cis) is elongated to 16:1(9, cis) and 18:1 (11, cis). Two condensation enzymes, β-ketoacyl-ACP synthetases I and II, are involved in the fatty acid synthesis. The synthetase I (coded for by fab B gene [109]) catalyzes the chain elongation of 12:0, 14:0, 12:1(5) and 14:1(7), while the synthetase II (coded for by fab F gene [110]) catalyzes preferentially the elongation of 16:1 to 18:1. The cyclopropane fatty acids are synthesized by transfer of a methyl group from S-adenosylmethionine to 16:1 and 18:1 [111] mainly at the stationary growth phase.

Phosphatidic acid (PA), a precursor of phospholipids, is synthesized from the acyl-CoA and glycerol-3-phosphate by two acyltransferases, glycerol-3-phosphate acyltransferase [112] and 1-acylglycerolphosphate acyltransferase [113]. The phospholipids are synthe-

sized from PA *via* two pathways: In one pathway, PA is converted to PS and then to PE, and in the other pathway, to PG and then to DPG [105].

When the fatty acid compositions are compared between cells grown at different temperatures, 16:0 decreases and 18:1 increases with decrease of the growth temperature [114,115]. Compositions of molecular species of PE and PG also depend on the temperature; saturated and monounsaturated molecular species decrease and diunsaturated molecular species increase with decrease of the temperature [116,117]. These changes in the lipid molecular species can be explained by temperature dependences of the fatty acid synthetase system [110, 118, 119] and of acyltransferase system [116]. Upon downward temperature shift, the unsaturation of newly synthesized lipids is affected, while the fatty acids that have been synthesized before the temperature shift are not desaturated. This is related to the lack of fatty acid desaturases in the bacterium.

The mode of thermo-adaptation in the synthesis of lipid molecular species seems to depend on the magnitude of temperature shift. Okuyama *et al.*[118] observed a "hyper-response" of synthesis of 18:1/18:1 species after temperature shift from 40 to 10°C. Nishihara *et al.* [116], on the other hand, did not see the "hyper-response" after temperature shift from 37 to 17°C.

The temperature-dependent changes in the fatty acid compositions of lipids are accompanied by modification of the thermal properties of membrane. The temperature for the onset of phase separation, T_f, shifts in parallel with the growth temperature [80, 81]. Sinensky, using a spin probe (methyl 12-nitroxystearate), found that viscosity of the membranes is constant when it is measured at the growth temperatures

[120]. Based on this fact, he [120] proposed a concept "homeoviscous adaptation", in which viscosity of the membrane lipids is kept constant at the growth temperature owing to the changes in molecular species composition of lipids. The regulation of membrane fluidity provides the membrane-bound enzymes with an adequate physical condition [88, 105, 121, 122].

(b) Brevibacterium ammoniagenes

This bacterium contains 16:0, 18:0 and 18:1 (9, cis). The relative content of 18:1 is higher at lower growth temperatures [123]. A fatty acid synthetase synthesizes all of these fatty acids. 18:1 is produced by the "anaerobic pathway" as in Escherichia coli. The relative rates of synthesis of fatty acids by the fatty acid synthetase depends on temperature. This can account for the growth temperature-dependent compositional changes in fatty acids in vivo [123].

(c) Bacilli

Bacilli contain normal and branched (iso and anteiso) fatty acids [124]. Monounsaturated acids are widely distributed in a wide variety of strains, while a diunsaturated acid, 16:2 (5,10), is found only in a limited number of strains [124]. The unsaturated fatty acids are synthesized from saturated ones by the "aerobic desaturation" (oxygen-dependent introduction of a double bond into a fatty acid chain), and the activity of desaturation depends on temperature. Bacillus megaterium synthesizes Δ5-monounsaturated fatty acids at 20°C but not at 30°C or above [125, 126].

Upon temperature shift from 30 to 20°C, the activity to introduce

a double bond at Δ5 position begins to increase after a short lag period (5 min), reaches the maximum level 60 min later, and then decreases to a level which is seen in the isothermal growth at 20°C [125, 127]. The transient increase in the desaturation activity, which is termed "hyperinduction" [125], is inhibited by rifampicin and chloramphenicol [127]. The increase is due to an induced synthesis of desaturase and the following decrease is explained by the action of repressor protein which is synthesized at 20°C [127].

Substrates of desaturation are believed to be acyl thioesters [128, 129]. A recent study [130], however, suggests that it might be phospholipid-bound fatty acids.

(d) Acholeplasma laidlawii

This parasitic microorganism changes its fatty acid composition with growth temperature [131]. The rates of incorporation of exogenously supplied 16:0 and 18:1 differ in their temperature dependence.

(2) Eukaryotic microorganisms

(a) Tetrahymena pyriformis

Tetrahymena contains 14:0, 16:0, 16:1, 16:2, 18:0, 18:1, 18:2 and 18:3(6,9,12) as major fatty acids [132, 133]. It synthesizes more 18:2 and 18:3 and less 16:0 and 18:0 when it is grown at lower temperatures [134-136]. After temperature shift from 39.5 to 15°C, 16:1 increases for 4 h and then decreases. 18:2 and 18:3 increase to attain stationary levels in 50 h [137]. The rapid increase in 16:1 after the temperature shift is explained by accelerated desaturation of 16:0 [137]. Two mechanisms have been proposed for the accelerated desaturation.

In one mechanism, a membrane-bound desaturase is activated by a temperature-induced alteration of physical state of membranes [138-140], and in the other one, a synthesis of 16:0-CoA desaturase is induced by the temperature shift [141-143]. A desaturase which converts 18:1-PC to 18:2-PC is induced by the downward temperature shift [144, 145]. This enzyme can account for the increase in 18:2 and 18:3 after the temperature shift.

(b) Yeasts

Yeasts are classified into two groups according to the fatty acid compositions. Those in the first group contain 16:0, 18:1, 18:2 and 18:3 as major components, whereas those in the second group contain 16:0, 16:1, 18:0 and 18:1 but not polyunsaturated acids [146]. The fatty acid synthetase of yeast is an enzyme complex having a molecular weight of 2.3×10^6 and is composed of two types of multifunctional polypeptides [107, 147]. The enzyme synthesizes the saturated fatty acids [148], from which the unsaturated fatty acids are synthesized by aerobic desaturation [146].

The effect of temperature on the fatty acid composition has been studied in the first group. The content of 18:2 is higher at lower temperatures in Candida lipolytica [149] and Candida utilis [150] In Candida lipolytica, the desaturation of 18:1 to 18:2 takes place in forms of 18:1-CoA and 18:1-PC [151, 152]. However, the high content of 18:2 at low temperatures is ascribed to the 18:1-CoA desaturase, which is more active than the 18:1-PC desaturase at low temperatures [151]. High activities of desaturations of 18:0-CoA [153] and 18:1-PC [153, 154] at low temperatures are demonstrated in Torulopsis utilis.

(c) Fungi

In Neurospora crassa, 18:0 is desaturated in the form of 18:0-CoA, while 18:1 is desaturated in the form of 18:1-PC [155]. The direct desaturation of 18:1-PC is demonstrated also in Fusarium oxysporum [156].

III. Aim of the present study

The aim of the present study is to elucidate the pathway of the biosynthesis of lipid molecular species in the blue-green alga, Anabaena variabilis. As the growth temperature is known to affect the compositions of lipid molecular species [8, 28, 96], the effect of growth temperature on the biosynthesis of lipid molecular species is also studied.

PC, a precursor of galactolipids in higher plants [51], does not exist in the blue-green algae [8, 15]. It is supposed, therefore, that the galactolipids are synthesized by a different pathway in the prokaryotic algae. In this respect, the discovery of GlcDG as a possible precursor of GalDG is of interest [11]. In Chapter 2, the experiments are conducted to elucidate the presence of GlcDG in Anabaena variabilis, and then the pathway of galactolipid synthesis is studied by pulse-labeling and chase experiments by using ^{14}C. The experimental results suggest that GlcDG is the primary product among the glycolipids, and that it is converted to GalDG which is, in turn, galactosylated to form Gal_2DG.

In Chapter 3, the biosynthesis of fatty acids and lipid molecular species is studied by pulse-labeling and chase experiments by using ^{14}C. Biosynthetic pathways of molecular species of glyco- and phospholipids are proposed. It is suggested there that the primary products are all saturated molecular species which are sequentially converted to unsaturated ones.

In Chapter 4, the effect of temperature shift on the biosynthesis of lipid molecular species is studied by ^{14}C-labeling. A characteristic modification of biosynthetic pathway of lipid molecular species is

discovered. A transient increase in the activity of a desaturation reaction after the temperature shift similar to the "hyperinduction" [125, 127] is demonstrated.

In Chapter 5, the mechanism of desaturation of 16:0 in GalDG is studied by ^{13}C-labeling and mass spectrometry. The experimental results definitely prove that 16:0 in GalDG is directly desaturated; in other words, the C_{16} acid remains esterified to GalDG when it is desaturated.

In Chapter 6, the mechanism of biosynthesis of lipid molecular species in Anabaena variabilis is discussed in comparison with the mechanisms in other microorganisms. A uniqueness in the temperature-acclimative regulation of lipid molecular species in this alga is described.

Chapter 2 Biosynthesis of lipid classes

I. SUMMARY

Lipid biosynthesis in intact cells of Anabaena variabilis was studied by ^{14}C-pulse-labeling and chase technique. The experimental results lead to the following conclusions. (1) Primary products of the lipid synthesis are GlcDG, SqDG and PG. (2) Monoglucosyl diacylglycerol (GlcDG) is converted to GalDG by epimerization of glucose unit. (3) A part of GalDG is further transformed to Gal_2DG by transfer of a galactose unit.

II. INTRODUCTION

There are two pathways of biosynthesis of galactolipids in higher plants (Fig. 2 in Chapter 1). In the first pathway, GalDG is synthesized in the chloroplast envelope from PC which is synthesized in the endoplasmic reticulum [51, 57], whereas, in the second pathway, it is synthesized by galactosylation of DG [63, 68]. Digalactosyl diacylglycerol (Gal_2DG) is synthesized from GalDG by dismutation of two molecules of GalDG [70] or transfer of a galactose unit to GalDG[68, 69].

In the blue-green algae, which lack PC, GalDG cannot be synthesized in the first pathway. In a study on the lipid synthesis in the blue-green algae, Appleby et al. [15] and Nichols [30] found that ^{14}C-acetate was incorporated into GalDG, Gal_2DG, SqDG and PG. Feige [31] and Feige et al.[11] discovered a small amount of GlcDG in a number of species of blue-green algae in a short-term labeling experiments using ^{14}C-bicarbonate, and inferred that GlcDG would be a precursor of GalDG.

In this chapter, the lipid biosynthesis in Anabaena variabilis

is studied by pulse-labeling and chase technique by using $NaH^{14}CO_3$. A detailed mechanism for the synthesis of glycolipids in this alga will be proposed.

III. MATERIALS AND METHODS

A. variabilis (strain M-3, in the Algal Culture Collection of the Institute of Applied Microbiology, University of Tokyo) was grown photoautotrophically at 22 and 38°C in Kratz and Myers' medium C [157] with aeration by 1 % CO_2 in air. The cultures were illuminated continuously by incandescent lamp with an intensity of 3000 lux for the growth at 22°C and 6000 lux for the growth at 38°C. The generation time was about 30 h at 22°C and 15 h at 38°C. Cells in the middle logarithmic phase (about 1 ml packed cell volume/liter culture) were used.

The algal cells were labeled with $NaH^{14}CO_3$ (30 to 40 μCi/ml, 1 mM) for 0.1 or 1.0 h under illumination of incandescent lamp with an intensity of 6000 lux. In some experiments, cerulenin (10 μg/ml) was added to the culture 0.1 h before the ^{14}C-labeling. In the chase for 10 h, the cells labeled for 1.0 h were washed twice with the fresh culture medium, and were incubated under the culture condition. In the chase for 0.9 h after the labeling for 0.1 h, non-radioactive $NaHCO_3$ was added after the labeling to a final concentration of 10 mM. The labeled cells were washed with and suspended in the fresh culture medium containing 10 mM $NaHCO_3$, and incubated in the light with aeration by air. All the procedures of labeling and chase were performed at the growth temperatures.

The lipids were extracted according to the procedure of Bligh and Dyer [158] and separated by TLC on plates coated with silica gel

(Merck 5721) which was developed in acetone/benzene/water (91:30:8, by vol.) [159]. The lipids were located by using a fluorescent dye, primuline [160]. Radioactivity on the silica gel plates was detected by autoradiography with an X-ray film (Kodak XR-5). When the radioactivity was quantitatively determined, silica gel in the radioactive zones was scraped into counting vials and the radioactivity was determined in 7 ml toluene containing 2,5-diphenyloxazole (4 g/liter) and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (0.1 g/liter) (Cocktail A) with a liquid scintillation spectrophotometer (Beckman LS-9000).

When intramolecular distribution of the radioactivity in the lipid molecules was determined, the lipids separated by TLC were methanolyzed in 3 % HCl/CH_3OH at 85°C for 2.5 h. The resultant fatty acid methyl esters were extracted with n-hexane and the radioactivity was determined by liquid scintillation counting in Cocktail A. The remaining methanol phase containing glycerol, methyl glycosides and phosphoric acid was concentrated by evaporation, and the radioactivity was determined in 7 ml toluene/Triton X-100 (2:1, v/v) containing 2,5-diphenyloxazole (6 g/liter) and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (0.5 g/liter) (Cocktail B). When the radioactivity in the sugar moiety of GlcDG and GalDG was determined, the lipids were methanolyzed, and the resultant methyl glycosides were isolated by TLC on plates coated with silica gel which was developed in acetone/benzene/water (91:30:8, by vol.). The radioactivity was detected by autoradiography. Silica gel in the radioactive zones was scraped off the plate, and the radioactivity was determined in Cocktail B.

The chemical structure of GlcDG was investigated by chemical analysis and mass spectrometry. For this purpose, GlcDG was extracted

from the unlabeled cells and purified by repeated TLC. For an analysis of chemical composition, the lipid was methanolyzed. The fatty acid methyl esters were extracted with n-hexane, and identified and determined by GLC with methyl pentadecanoate as an internal standard. A gas chromatograph (Shimadzu GC-6AM) equipped with a H_2 ionization detector and a column (2 m x 3 mm i.d.) packed with 15 % diethyleneglycol succinate on Chromosorb W (acid washed and DMCS-treated) was used. Column temperature was 185°C. The flow rate of N_2 carrier gas was 30 ml/min. Methyl glycoside and glycerol recovered from the methanol phase were trimethylsilylated with a mixture of hexamethyldisilazane and trimethylchlorosilane. Glucose and glycerol were identified by GLC with a column (2 m x 3 mm i.d.) packed with 5 % silicone SE-30 on Chromosorb W (acid washed and DMCS-treated). Column temperature was first 140°C for 3 min and then increased to 200°C at a rate of 3°C/min. The flow rate of N_2 carrier gas was 30 ml/min. The amounts of methyl glucoside and glycerol were determined by the same GLC with mannitol as an internal standard [161]. For the mass spectrometric analysis, the lipid was trimethylsilylated with trimethylsilylimidazol, and applied to a gas chromatography-mass spectrometer (Shimadzu-LKB 9000B) with a column (0.5 m x 3 mm i.d.) packed with 1.5 % OV-1 on Chromosorb W (acid washed and DMCS-treated) operated at 270°C. The flow rate of He carrier gas was 50 ml/min. Mass spectra were taken at an ionization voltage of 20 eV.

$NaH^{14}CO_3$ (59-55 Ci/mol) was obtained from Radiochemical Centre (Amersham, U. K.) and CEA (Saclay, France). Cerulenin was from Makor Chemicals (Jerusalem, Israel). All the organic solvents and other chemicals were from Wako Pure Chemical Industries (Osaka, Japan)

and Tokyo Chemical Industry (Tokyo, Japan). All the solvents were of special grade and used without further purification.

IV. RESULTS

Figure 3 shows the autoradiograms of TLC of lipids extracted from the algal cells which were labeled with $NaH^{14}CO_3$ at 22°C. The radioactivity was found in all of the four lipid classes abundant in the blue-green alga. Chlorophyll a and carotenoids were also labeled. Free fatty acids contained less than 0.5 % of the total radioactivity in the lipids. A lipid which migrated a little faster than GalDG was highly radioactive after the labeling for 0.3 and 1.0 h. However, the radioactivity disappeared after the chase for 10 h. In respect of the chromatographic behavior, this lipid is very similar to "X-MGD" discovered by Feige [11] in a number of blue-green algae.

In order to study the chemical structure of the lipid, we used the unlabeled one, which was extracted from cells grown at 22°C and purified by repeated TLC. The lipid was methanolyzed, and the products were analyzed by GLC. The experimental result indicated that there were glycerol, methyl glucoside and fatty acid methyl esters in a molar ratio of 0.96 : 1 : 2.17. This fact suggests that a molecule of the lipid contains one glycerol, one glucose and two fatty acid molecules. In another experiment, the lipid was compared with GalDG by GLC and mass spectrometry as trimethylsilyl derivatives. In the GLC on SE-30, a major peak of the lipid appeared at 20 min, whereas that of GalDG appeared at 19 min. A mass spectrum of the derivative of the lipid showed prominent fragments characteristic of hexose (m/e 204, 217 and 361) and diacylglycerol (m/e 577 corresponding to

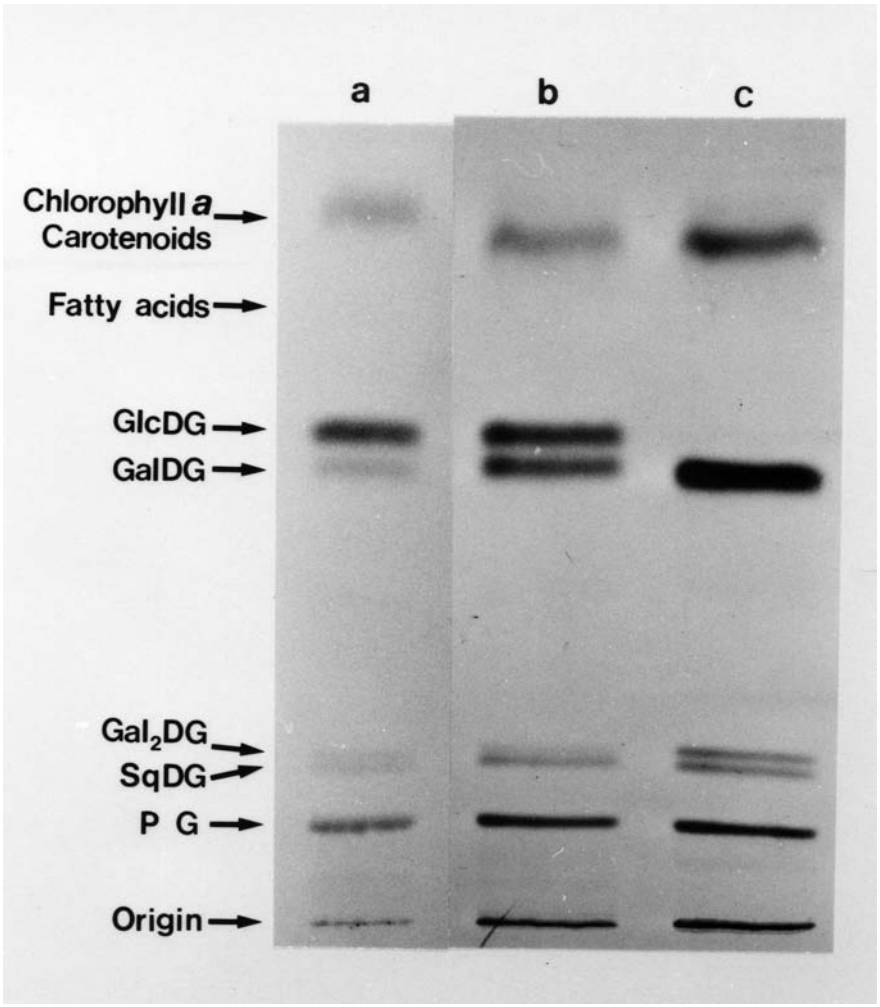

Fig. 3 Autoradiogram of TLC of lipids from A. variabilis grown and labeled with $NaH^{14}CO_3$ at 22°C.
a, ^{14}C-labeling for 0.3 h; b, ^{14}C-labeling for 1.0 h;
c, ^{14}C-labeling for 1.0 h followed by a chase for 10 h.

(blank page)

oleoylpalmitoylglycerol). Other peaks appeared at about m/e 1044, 1029 and 939, although the m/e values of these peaks could not be accurately determined because of limited resolution in the high mass region. The peaks would correspond to the molecular ion, M^+, and the fragment ions, $(M-15)^+$ and $(M-105)^+$. The mass spectra of the trimethylsilyl derivatives of GlcDG and GalDG were virtually identical. These findings confirm the structure of monoglucosyl diacylglycerol (GlcDG), which was proposed by Feige et al.[11] on the basis of the mass spectrum of the acetylated derivative of the lipid and the susceptibility to glycosidases of a deacylated derivative of the lipid.

Table 1 shows distribution of the radioactivity among the lipid classes after the labeling with $NaH^{14}CO_3$ for 0.1 and 1.0 h, and the labeling for 1.0 h followed by a subsequent chase for 10 h. Upon the labeling for 0.1 h at 22°C, most of the radioactivity was found in GlcDG and PG. Sulfoquinovosyl diacylglycerol (SqDG), GalDG and Gal_2DG were only slightly labeled. During the labeling for 1.0 h, the relative radioactivity markedly decreased in GlcDG and increased in GalDG. During the subsequent chase for 10 h, GlcDG lost the radioactivity, whereas GalDG and Gal_2DG gained it. A precursor-product relationship between GlcDG and GalDG was seen. These results are compatible with the suggestion by Feige et al. [11] that GlcDG is a precursor of GalDG. The relative radioactivity in PG was high after the labeling for 0.1 h, but decreased during the labeling for 1.0 h as well as the subsequent chase for 10 h. At the end of the chase, the distribution of radioactivity among the lipid classes was close to the mass distribution.

Similar but slightly different labeling patterns were obtained at

Table 1. Distribution of radioactivity among lipid classes after the pulse-labeling with $NaH^{14}CO_3$ and the subsequent chase. Cells grown at 22 and 38°C were pulse-labeled with $NaH^{14}CO_3$ and chased at 22 and 38°C, respectively.

Labeling		Chase	Radioactivity (%)					Total radioactivity (kcpm)	
°C	h	h	GlcDG	GalDG	Gal_2DG	SqDG	P G	Lipids	Cells
22	0.1	---	58	4	4	6	28	29	-----
22	1.0	---	34	30	4	7	25	457	-----
22	1.0	10	1	61	7	8	23	595	11755
22	(Mass)*		(1)	(55)	(17)	(11)	(17)		
38	0.1	---	34	7	6	13	40	51	1459
38	1.0	---	19	41	4	9	27	1062	12229
38	1.0	10	0	54	12	11	23	1331	13104
38	(Mass)*		(1)	(52)	(19)	(14)	(14)		

* Mass compositions determined as in [8] were expressed on the basis of carbon atoms.

38°C (Table 1). During the labeling for 1.0 h, the relative radioactivity of GalDG increased faster than that at 22°C. The relative labeling of PG at 0.1 h and of Gal_2DG after the chase for 10 h was higher at 38°C than at 22°C. These findings suggest that the lipids are synthesized essentially in similar ways at 22 and 38°C and that the turnover of PG and the synthesis of Gal_2DG are faster at 38°C.

Intramolecular distribution of the radioactivity in the lipid classes was investigated. Table 2 shows the relative radioactivity in the sugar-and-glycerol or glycerol moiety. No great difference was found between the results obtained at 22 and 38°C. The relative radioactivity localized in the sugar-and-glycerol part ranged from 0.21 to 0.27 in GlcDG, GalDG and SqDG. The relative radioactivity localized in the glycerol part ranged from 0.18 to 0.21 in PG. These values were consistent with the intramolecular distribution of carbon atoms in the corresponding lipid classes. The intramolecular distribution of radioactivity remained almost constant during the chase for 10 h in these lipid classes.

On the other hand, the sugar-and-glycerol part of Gal_2DG contained the radioactivity as much as 60 to 70 % of the total when the cells were labeled for 0.1 and 1.0 h. The relative radioactivity is about twice as much as the relative content of carbon atoms in the digalactosylglycerol moiety. The specific radioactivity was about four times* as high in

---

* The sugar-and-glycerol and the fatty acid parts of Gal_2DG contain 15 and 34 carbon atoms, respectively. The specific radioactivities in the sugar-and-glycerol and fatty acid parts, in an arbitrary unit, are (0.65/15) and (0.35/34), respectively.

Table 2. Relative radioactivity in sugar-and-glycerol moiety or in glycerol moiety of the lipids. Experimental procedures of the labeling were the same as in Table 1.

Labeling		Chase	Radioactivity in sugar-and-glycerol moiety*				
°C	h	h	GlcDG	GalDG	Gal_2DG	SqDG	P G
22	0.1	---	0.27	0.24	0.60	0.24	0.21
22	1.0	---	0.22	0.23	0.66	0.25	0.18
22	1.0	10	ND**	0.26	0.44	0.24	0.18
38	0.1	---	0.24	0.21	0.68	0.21	0.20
38	1.0	---	0.23	0.23	0.70	0.23	0.18
38	1.0	10	ND**	0.26	0.44	0.24	0.18
Distribution of carbon atoms in lipid molecule***			0.21	0.21	0.31	0.21	0.15

* In the glyceroglycolipids, the radioactivity in the sugar-and-glycerol moiety divided by the radioactivity in the whole molecule. In PG, the radioactivity in the glycerol moiety divided by the radioactivity in the whole molecule.

** ND, not determined.

*** Calculated on the basis that the lipid molecule contains 1 mole of C_{16} and 1 mole of C_{18} acid [8].

the digalactosylglycerol moiety as in the acyl groups. During the chase for 10 h, the intramolecular distribution of radioactivity in this lipid decreased toward that of carbon atoms. This change can be interpreted as an increase in the specific radioactivity in the fatty acid part.

An addition of cerulenin, an inhibitor of fatty acid synthetase [162], almost completely inhibited the incorporation of ^{14}C into GlcDG, GalDG, SqDG and PG, but far less effectively into Gal_2DG (Table 3). These results indicate that GlcDG, SqDG and PG are produced only when the fatty acids are supplied by de novo synthesis.

When the distribution of radioactivity was analyzed in Gal_2DG, cerulenin was found to inhibit the labeling in the fatty acid part but not at all in the galactose-and-glycerol part. This fact suggests that the newly synthesized fatty acids are not necessary for the synthesis of the lipid. This is consistent with the mechanism established in higher plants in which Gal_2DG is synthesized by galactosylation of GalDG. [163, 68, 69].

The conversion of GlcDG to GalDG was studied in more detail by a short-term pulse-labeling and chase experiment. Figure 4a shows changes in the radioactivity in GlcDG and GalDG when the cells were labeled for 0.1 h and chased for 0.9 h at 22°C. The radioactivity in GlcDG continued to increase for 0.2 h after the labeling and then decreased. The radioactivity in GalDG, on the other hand, increased throughout the chase period. The total radioactivity in the two lipids remained almost constant from 0.2 to 0.9 h. These findings indicate that most of GlcDG is converted to GalDG in 1 h at 22°C.

Figure 4b shows changes in the radioactivity in the sugar moiety

Table 3. Effect of cerulenin on the incorporation of ^{14}C into lipids. Cells grown at 22°C were labeled with $NaH^{14}CO_3$ at 22°C for 1.0 h with or without cerulenin (10 μg/ml).

	Radioactivity (cpm)							
			Gal_2DG					
Cerulenin	GlcDG	GalDG	Total	FA*	Gal+Gly*	SqDG	P G	Total
-	32400	31100	3450	(1170)	(2280)	9180	28400	104500
+	180	1220	2620	(230)	(2390)	520	340	4880
Relative incorporation (%)	1	4	76	(20)	(105)	6	1	5

* Abbreviations: FA, fatty acids; Gal+Gly, galactose and glycerol.

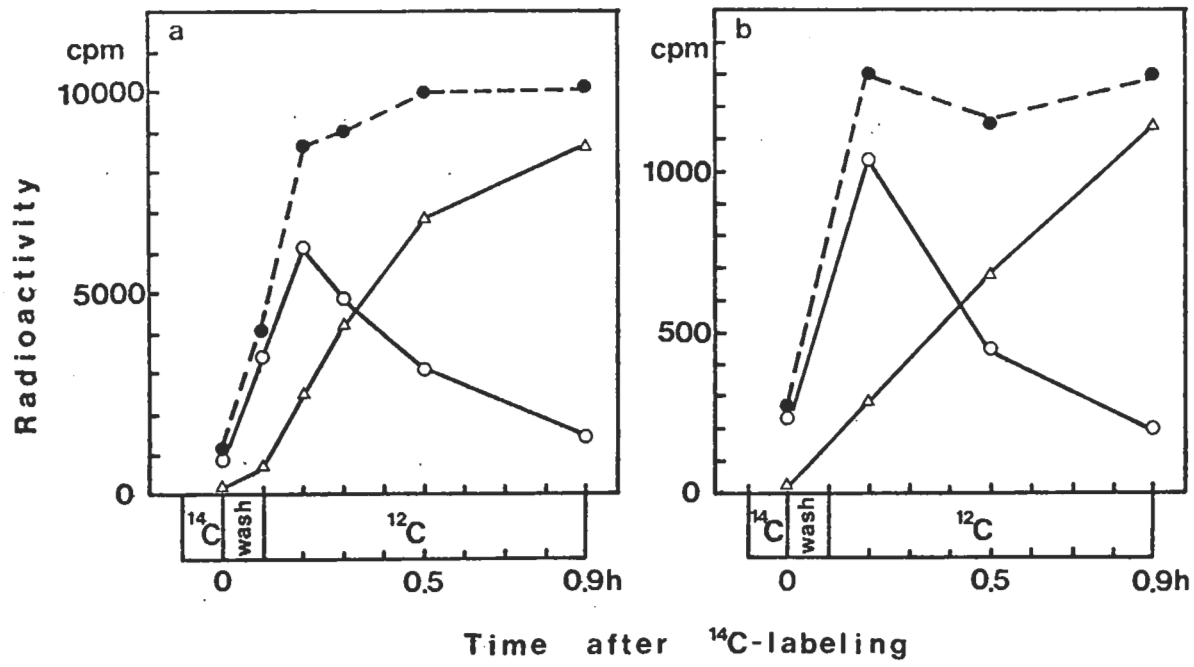

Fig. 4. Changes in radioactivity in GlcDG and GalDG during pulse-labeling and chase. The cells grown at 22°C were labeled with $NaH^{14}CO_3$ for 0.1 h and then incubated with cold $NaHCO_3$.
a, GlcDG (○), GalDG (△) and total (●).
b, glucose in GlcDG (○), galactose in GalDG (△) and total (●).

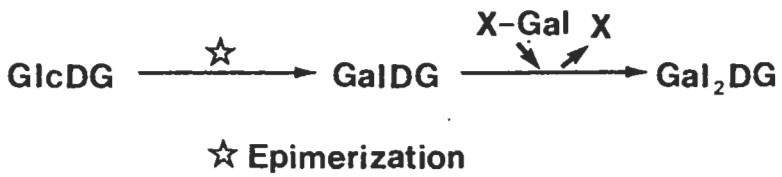

Fig. 5. A proposed scheme for the pathway of biosynthesis of glycolipids in A. variabilis. X is a galactose carrier, possibly uridine diphosphate.

of GlcDG and GalDG during the chase after the labeling for 0.1 h. From 0.2 to 0.9 h, the radioactivity in glucose unit of GlcDG decreased and that in galactose unit of GalDG increased. The total radioactivity in the sugar moiety of the two lipids remained almost constant during the chase period, indicating that the radioactivity in the sugar moiety was not lost during the conversion of GlcDG to GalDG. These results suggest that glucose in GlcDG is a precursor of galactose in GalDG.

V. DISCUSSION

In the pathway of glycolipid biosynthesis in A. variabilis elucidated in this study, the primary product is GlcDG, which is converted to GalDG. A part of the latter lipid is further converted to Gal_2DG. The mechanism of synthesis of GalDG in the blue-green alga is different from either of the two mechanisms in higher plants in which GalDG is synthesized either from PC [51, 57] or from DG [63, 68, 163]. The content of GlcDG in A. variabilis, about 1 % of the total lipids (Table 1), is much higher than those in other blue-green algal species (less than 0.1 % of the total lipids) examined by Feige et al.[11].

There are two possible mechanisms for the conversion of GlcDG to GalDG. One is an exchange of the glucose unit by a newly synthesized galactose unit, and the other is epimerization at the 4'-carbon atom of glucose unit. Figure 4 shows that the radioactivity in the sugar moiety remains almost constant during the chase, when GlcDG is converted to GalDG. Table 3 shows that cerulenin, which inhibits the synthesis of fatty acids but not of sugars, almost completely blocks the labeling of GalDG. These findings are compatible with the latter mechanism.

If the former mechanism had taken place, almost none of the radioactivity in the glucose unit of GlcDG should have been shifted to the galactose unit of GalDG, and the labeling of galactose unit of GalDG should not have been fully inhibited by cerulenin. We conclude, therefore, that GlcDG is transformed to GalDG by the epimerization of glucose to galactose in the lipid.

The experimental results suggest that Gal_2DG is synthesized from GalDG. Two mechanisms have been proposed for the synthesis of Gal_2DG in higher plants. Ongun and Mudd [68], and Siebertz and Heinz [69] infer that UDP-galactose donates the galactose unit to GalDG to form Gal_2DG. Van Besouw and Wintermans [70], on the other hand, claim that dismutation of two GalDG molecules produces one Gal_2DG and one DG molecules. The experimental result in A. variabilis that cerulenin does not affect the labeling of digalactosylglycerol moiety of Gal_2DG (Table 3) is compatible with the mechanism in which the newly synthesized galactose is transferred to GalDG to form Gal_2DG. If Gal_2DG had been produced by the dismutation of two GalDG molecules, i.e., the transfer of galactose unit from a GalDG molecule to another, the labeling of the digalactosylglycerol moiety of Gal_2DG should have been markedly reduced in the presence of cerulenin, which blocked the labeling of GalDG. We conclude, therefore, that Gal_2DG is formed from GalDG by the transfer of a newly synthesized galactose unit. Whether the donor of the galactose unit is UDP-galactose is still in question. The pathway of glycolipid synthesis in A. variabilis is presented in Fig. 5.

The present study shows that SqDG and PG are synthesized de novo. The significantly high content of radioactivity in PG after the labeling for 0.1 h made us to suspect that PG turns over faster than the other

lipids, or that it is a precursor of cellular constituents other than the lipids.

Chapter 3 Biosynthesis of lipid molecular species

I. SUMMARY

Biosynthesis of lipid molecular species was studied in Anabaena variabilis by ^{14}C-pulse-labeling and chase technique. The experimental results indicate that the primary products of lipid biosynthesis are 1-stearoyl(18:0)-2-palmitoyl(16:0) molecular species of GlcDG, PG and SqDG. In GlcDG, 18:0 is rapidly converted to 18:1 and further to 18:2, whereas 16:0 is hardly converted to 16:1. Monoglucosyl diacylglycerol (GlcDG) is converted to GalDG when the combination of acyl groups are either 18:0/16:0, 18:1/16:0 or 18:2/16:0. Further desaturation of the C_{16} and C_{18} acids takes place in GalDG, in which at 38°C, 18:0/16:0 molecular species is converted to 18:1/16:0, then to either 18:2/16:0 or 18:1/16:1, and finally to 18:2/16:1 molecular species, and at 22°C, the 18:0/16:0 molecular species is sequentially converted to 18:1/16:0, 18:2/16:0, 18:3/16:0 and 18:3/16:1 molecular species. The molecular species of Gal_2DG are synthesized from the corresponding molecular species of GalDG. Desaturation does not seem to occur in Gal_2DG.

In PG and SqDG, 18:0 is desaturated to 18:1 and 18:2 at 38°C and further to 18:3 as well at 22°C. 16:0 is hardly desaturated in these classes of lipids.

II. INTRODUCTION

In higher plants, 18:0 is desaturated to 18:1 when it is esterified to ACP [54]. The produced 18:1 is incorporated into PC or GalDG and then desaturated to 18:2 [61, 63, 64, 65]. The phosphocholine group of dilinoleoyl-PC is replaced by galactosyl group to form dilinoleoyl-

monogalactosylglycerol [55]. In the GalDG-bound form, 18:2 is further desaturated to 18:3 [51, 61, 63].

In blue-green algae, in which GalDG is synthesized from GlcDG (Chapter 2), the mechanism of desaturation of fatty acids is to be different from that in higher plants. Nichols [30] has shown a shift of radioactivity from saturated to unsaturated fatty acids in complex lipids in the blue-green algae, Anabaena cylindrica and Anacystis nidulans. Appleby et al.[15] have suggested that 18:1 in GalDG is desaturated to 18:2 in Anabaena variabilis. When these studies were published, the presence and the function of GlcDG was not known. The role of this lipid in the fatty acid desaturation in the blue-green algae is now in question. In this chapter, desaturation of the fatty acids in lipids and biosynthesis of the lipid molecular species in the blue-green alga, A. variabilis are studied.

III. MATERIALS AND METHODS

Culture conditions, labeling procedures and methods of lipid isolation were the same as those described in Chapter 2.

The isolated lipids were methanolyzed as in Chapter 2, and the fatty acid methyl esters were analyzed by radio-GLC (Shimadzu model GC-4B) with a glass column (1.5 m x 3 mm i.d.) packed with 15 % EGSS-X on Chromosorb W. The column temperature was 160°C and the flow rate of He carrier gas was 60 ml/min.

Molecular species of the glycolipids were separated by argentation TLC. Precoated silica gel plates (Merck 5721) were impregnated with $AgNO_3$ by immersing it in 10 % $AgNO_3$ in acetonitrile for 30 min and activating it at 130°C for 30 min. Developing solvents in TLC were

acetone/benzene/water (91:30:8, by vol.)[159] for GlcDG and GalDG and chloroform/methanol/water (60:30:4, by vol.)[63] for Gal_2DG. On the other hand, PG was treated with phospholipase C (EC 3.1.4.3) and the resultant diacylglycerol was acetylated in acetic anhydride/pyridine (2:1, v/v) [164]. Molecular species of the monoacetyl diacylglycerol were separated by argentation TLC with benzene/chloroform/methanol (98:2:0.1, by vol.) [164] as developing solvent. The molecular species were identified by analyzing fatty acids at the C-1 and C-2 positions by using a lipase (EC 3.1.1.3) from Rhizopus delemar [8, 28]. Radioactivity was detected by autoradiography and measured by liquid scintillation counting as described in Chapter 2. In the argentation TLC, all the molecular species listed in Tables 5, 7, 9 and 11 were separated from one another.

Mass compositions of the fatty acids in lipids were determined by GLC as described in Chapter 2. Mass compositions of the molecular species of GalDG and Gal_2DG were determined on the basis of the amounts of fatty acids in the molecular species separated by the argentation TLC. For determination of mass composition of the molecular species of PG, monoacetyl diacylglycerol derived from PG was directly applied to GLC (Shimadzu GC-6AM) equipped with a H_2 ionization detector. They were separated on a glass column (2 m x 3 mm i.d.) packed with 2 % SILAR 10C on Uniport HP [165,166]. The column temperature was programmed to increase from 250 to 270°C at a rate of 1°C/min, and the flow rate of N_2 carrier gas was 40 ml/min.

Intramolecular distribution of radioactivity in lipids was determined by methanolysis as described in Chapter 2.

Phospholipase C was obtained from Calbiochem-Behring Corp. (La Jolla, U. S. A.). The lipase from Rhizopus delemar was a product of

Seikagaku Kogyo Co. (Tokyo, Japan). The sources of other reagents and solvents were the same as those in Chapter 2.

IV. RESULTS

Monoglucosyl diacylglycerol (GlcDG)

Table 4 shows distribution of the radioactivity among the fatty acids esterified to GlcDG after the labeling with $NaH^{14}CO_3$. Upon the labeling at 38°C for 0.1 h, the radioactivity was incorporated into 16:0, 18:0 and 18:1. During the labeling for 1.0 h, the relative radioactivity decreased in 18:0 and increased in 18:1 and 18:2. The radioactivity scarcely shifted from 16:0 to 16:1. A similar result was obtained at 22°C. The radioactivity, however, shifted from 18:0 to 18:1 and 18:2 more rapidly at this temperature. It is noted that a distinct, but very small amount of radioactivity was found in 18:3 after the labeling for 1.0 h. The distribution of the radioactivity after the labeling for 1.0 h was similar to the mass composition of fatty acids. The distribution of the radioactivity after the chase for 10 h could not be determined, since almost all of the radioactivity in this class of lipid had already shifted to GalDG (Chapter 2).

Table 5 shows distribution of the radioactivity among the molecular species of GlcDG. At 38°C, most of the radioactivity was found in 18:0/16:0 after the labeling for 0.1 h and in 18:1/16:0 after the labeling for 1.0 h. The radioactivity was incorporated also into 18:2/16:0 during the labeling for 1.0 h. A similar result was obtained at 22°C. The shift of the radioactivity to the polyunsaturated molecular species was, however, faster at 22°C. The radioactivity was detected in 18:3/16:0, though in a small amount, after the labeling for

Table 4. Changes in distribution of the radioactivity among the fatty acids esterified to GlcDG.

Labeling		Chase	Percentage radioactivity							Total radio-activity in fatty acids
°C	h	h	16:0	16:1	16:2	18:0	18:1	18:2	18:3	(kcpm)
38	0.1	--	53	0	0	30	17	0	0	13
38	1.0	--	49	2	0	4	40	5	0	154
38	1.0	10	--	--	--	--	--	--	--	Trace*
38	Mass		(51)	(2)	(0)	(12)	(30)	(5)	(0)	
22	0.1	--	53	0	0	24	23	0	0	12
22	1.0	--	50	2	0	5	29	13	1	128
22	1.0	10	--	--	--	--	--	--	--	Trace*
22	Mass		(50)	(3)	(0)	(11)	(20)	(12)	(3)	

*Trace, less than 0.5 kcpm.

Table 5 . Changes in distribution of the radioactivity among the molecular species of GlcDG. GlcDG was directly applied to argentation TLC and the radioactivity of each separated molecular species was determined. Thus, the determined radioactivity includes the radioactivity in glycerol, glucose and fatty acids.

Labeling °C	Labeling h	Chase h	Percentage radioactivity C-1 / C-2								Total radioactivity in the lipid (kcpm)
			18:0 16:0	18:1 16:0	18:2 16:0	18:1 16:1	18:3 16:0	18:2 16:1	18:3 16:1	18:3 16:2	
38	0.1	--	70	30	0	0	0	0	0	0	17
38	1.0	--	10	74	17	0	0	0	0	0	200
38	1.0	10	--	--	--	--	--	--	--	--	Trace*
38	Mass**		(24)	(60)	(10)	(0)	(0)	(0)	(0)	(0)	
22	0.1	--	52	48	0	0	0	0	0	0	17
22	1.0	--	12	56	29	0	3	0	0	0	164
22	1.0	10	--	--	--	--	--	--	--	--	Trace*
22	Mass**		(22)	(40)	(24)	(0)	(3)	(0)	(0)	(0)	

* Trace, less than 0.5 kcpm.

** Estimated from the mass composition of fatty acids in Table 4.

1.0 h. These results suggest that GlcDG is synthesized in the form of 18:0/16:0, which is immediately converted to 18:1/16:0. A part of 18:1/16:0 is further converted to 18:2/16:0 and, at 22°C, to 18:3/16:0.

Monogalactosyl diacylglycerol (GalDG)

The radioactivity in GalDG was very low upon the ^{14}C-labeling for 0.1 h, but increased markedly during the continued labeling for 1.0 h (Tables 6 and 7). This increase is due to a conversion of GlcDG to GalDG (Chapter 2).

Table 6 shows distribution of the radioactivity among the fatty acids esterified to GalDG. The distribution of the radioactivity after the labeling for 0.1 h could not be accurately determined because of a low level of radioactivity in this class of lipid. Nevertheless, occurrence of the radioactivity in 16:0, 18:0 and 18:1 was noticed.

After the labeling for 1.0 h at 38°C, most of the radioactivity was found in 16:0 and 18:1. A small amount of radioactivity was detected in 16:1, 18:0 and 18:2. The distribution after the labeling for 1.0 h at 38°C was similar to that in GlcDG. During the subsequent chase for 10 h, most of the radioactivity in 16:0 and 18:1 shifted to 16:1 and 18:2. The distribution after the chase was similar to the mass composition of fatty acids in GalDG.

After the labeling for 1.0 h at 22°C, most of the radioactivity was found in 16:0, 18:1 and 18:2. A small amount of radioactivity was found in 16:1 and 18:3. During the chase for 10 h, most of the radioactivity in C_{18} acids shifted to 18:3. The shift of radioactivity from 16:0 to 16:1, however, was slower at 22°C than at 38°C. Hexadecadienoic acid (16:2), which amounted to 6 % in the mass composition at 22°C [8, 28],

Table 6. Changes in distribution of the radioactivity among the fatty acids esterified to GalDG.

Labeling		Chase	Percentage radioactivity							Total radio-activity in fatty acids (kcpm)
°C	h	h	16:0	16:1	16:2	18:0	18:1	18:2	18:3	
38	0.1	--	+	--	--	+	+	--	--	2.7
38	1.0	--	49	4	0	5	39	3	0	339
38	1.0	10	24	28	1	0	14	34	0	543
38	Mass		(26)	(25)	(.6)	(.3)	(19)	(28)	(.3)	
22	0.1	--	+	--	--	+	+	--	--	0.9
22	1.0	--	46	4	0	1	23	21	5	106
22	1.0	10	30	17	2	0	1	7	44	269
22	Mass		(26)	(20)	(6)	(.1)	(2)	(6)	(40)	

+, radioactivity present.

Table 7. Changes in distribution of the radioactivity among the molecular species of GalDG. GalDG was directly applied to argentation TLC and the radioactivity of each separated molecular species was determined. Thus, the determined radioactivity includes the radioactivity in glycerol, galactose and fatty acids.

Labeling		Chase	Percentage radioactivity								Total radioactivity in the lipid (kcpm)
			C-1	18:0	18:1	18:2	18:1	18:3	18:2	18:3	18:3	
°C	h	h	C-2	16:0	16:0	16:0	16:1	16:0	16:1	16:1	16:2	
38	0.1	--		55	45	0	0	0	0	0	0	3.4
38	1.0	--		8	75	12	4	0	0	0	0	440
38	1.0	10		0	20	33	13	1	34	0	0	725
38	Mass			(1)	(25)	(23)	(11)	(1)	(35)	(0)	(0)	
22	0.1	--		61	39	0	0	0	0	0	0	1.2
22	1.0	--		5	42	42	0	8	3	0	0	138
22	1.0	10		0	3	7	0	46	4	34	5	363
22	Mass			(2)	(2)	(12)	(0)	(34)	(3)	(32)	(12)	

contained only 1 % of the total radioactivity.

Table 7 shows distribution of the radioactivity among the molecular species of GalDG. After the labeling for 0.1 h at 38°C, 18:0/16:0 and 18:1/16:0 were the most radioactive species. After the labeling for 1.0 h, 18:1/16:0 was the most radioactive. Low radioactivity was found in 18:0/16:0, 18:2/16:0 and 18:1/16:1. During the chase for 10 h, most of the radioactivity in 18:1/16:0 shifted to 18:2/16:0, 18:1/16:1 and 18:2/16:1. These results were consistent with the distribution of radioactivity among the fatty acids esterified to GalDG (Table 6).

Intramolecular distribution of the radioactivity in 18:2/16:0, 18:1/16:1 and 18:2/16:1 after the chase for 10 h were examined. In all of the molecular species, the fatty acids contained about 80 % of the total radioactivity in the lipid molecule. This finding together with the previous one (Table 2 in Chapter 2) suggests that the polyunsaturated molecular species are synthesized from the saturated and monounsaturated molecular species by desaturation of the acyl groups.

Upon the labeling for 0.1 h at 22°C, the radioactivity was incorporated into 18:0/16:0 and 18:1/16:0, and, after the labeling for 1.0 h, 18:1/16:0 and 18:2/16:0 were equally labeled. Only a small amount of radioactivity was found in 18:0/16:0, 18:3/16:0 and 18:2/16:1. During the chase for 10 h, the radioactivity shifted to 18:3/16:0, 18:3/16:1 and 18:3/16:2. It is noted that 18:1/16:1 was not labeled at 22°C.

Digalactosyl diacylglycerol (Gal_2DG)

Table 8 shows distribution of the radioactivity among the fatty acids esterified to Gal_2DG. After the labeling for 0.1 h, Gal_2DG was hardly labeled with ^{14}C. After the labeling for 1.0 h at 38°C, the

Table 8. Changes in distribution of the radioactivity among the fatty acids esterified to Gal_2DG.

Labeling		Chase	Percentage radioactivity							Total radioactivity in fatty acids (kcpm)
°C	h	h	16:0	16:1	16:2	18:0	18:1	18:2	18:3	
38	0.1	--	--	--	--	--	--	--	--	1.2
38	1.0	--	50	0	0	31	14	5	0	13
38	1.0	10	29	20	1	2	22	24	2	123
38	Mass		(22)	(29)	(1)	(.2)	(14)	(33)	(1)	
22	0.1	--	--	--	--	--	--	--	--	0.5
22	1.0	--	42	1	0	21	29	6	0	6
22	1.0	10	32	15	2	0	3	19	29	27
22	Mass		(21)	(24)	(7)	(.3)	(1)	(7)	(40)	

Table 9. Changes in distribution of the radioactivity among the molecular species of Gal_2DG. Gal_2DG was directly applied to argentation TLC and the radioactivity of each separated molecular species was determined. Thus, the determined radioactivity includes the radioactivity in glycerol, galactose and fatty acids.

Labeling		Chase		Percentage radioactivity								Total	
°C	h	h	C-1 C-2	18:0 16:0	18:1 16:0	18:2 16:0	18:1 16:1	18:3 16:0	18:2 16:1	18:3 16:1	18:3 16:2	R.A.*	(FA/lipid)**
38	0.1	--		--	--	--	--	--	--	--	--	3.6	0.32
38	1.0	--		9	33	18	12	0	25	3	0	42	0.30
38	1.0	10		1	27	25	16	0	27	2	0	196	0.56
38	Mass			(1)	(16)	(24)	(16)	(0)	(38)	(0)	(0)		
22	0.1	--		--	--	--	--	--	--	--	--	1.2	0.40
22	1.0	--		8	26	9	0	20	2	24	11	19	0.34
22	1.0	10		0	5	22	0	35	6	25	7	48	0.56
22	Mass			(1)	(4)	(20)	(1)	(19)	(9)	(37)	(4)		

* Total radioactivity in the lipid (kcpm).

** Radioactivity in the fatty acids divided by radioactivity in the whole molecule, taken from Table 2 in Chapter 2.

radioactivity was found in 16:0, 18:0 and 18:1. The relative radioactivity in 18:0 was far greater than those in GlcDG and GalDG. At the end of the subsequent chase for 10 h, the radioactivity was distributed among 16:0, 16:1, 18:1 and 18:2.

A very similar result was obtained at 22°C. After the labeling for 1.0 h, the radioactivity was found in 16:0, 18:0 and 18:1. During the chase for 10 h, the radioactivity shifted to 16:1, 18:2 and 18:3. The labeled fatty acids in Gal_2DG were less desaturated than those in GalDG.

Distribution of the radioactivity among the molecular species of Gal_2DG was studied (Table 9). After the labeling for 1.0 h at 38 and 22°C, the radioactivity was distributed among all of the molecular species which were present in the mass composition. It is noted that 18:2/16:1 and 18:3/16:1 molecular species were densely labeled at 38 and 22°C, respectively (Table 9), although 18:2, 18:3 and 16:1 were scarcely labeled (Table 8). Analysis of the intramolecular distribution of the radioactivity indicated that 90 % of the total radioactivity in the molecule was localized in the digalactosylglycerol moiety in these polyunsaturated molecular species. During the chase for 10 h, the relative radioactivity decreased in 18:0/16:0 and 18:1/16:0, and increased in 18:2/16:0 at 38 and 22°C. An increase in 18:3/16:0 was also seen at 22°C. These changes were qualitatively consistent with those found in the fatty acids (Table 8). A similar labeling of polyunsaturated molecular species of Gal_2DG is noticed in higher plants[60, 63].

Phosphatidylglycerol (PG)

Table 10 shows distribution of the radioactivity among the fatty

Table 10. Changes in distribution of the radioactivity among the fatty acids esterified to PG.

Labeling		Chase	Percentage radioactivity							Total radio-activity in fatty acids (kcpm)
°C	h	h	16:0	16:1	16:2	18:0	18:1	18:2	18:3	
38	0.1	--	51	0	0	40	9	0	0	18
38	1.0	--	49	1	0	12	33	4	0	196
38	1.0	10	51	2	0	2	23	22	0	199
38	Mass		(51)	(3)	(0)	(2)	(25)	(19)	(.1)	
22	0.1	--	48	0	0	28	24	0	0	6
22	1.0	--	45	2	0	4	33	16	0	89
22	1.0	10	48	4	0	0	2	11	35	108
22	Mass		(54)	(2)	(0)	(1)	(3)	(8)	(32)	

Table 11. Changes in distribution of the radioactivity among the molecular species of PG. Monoacetyl diacylglycerol derived from PG was separated by argentation TLC and the radioactivity of separated molecular species was determined. Thus, the determined radioactivity includes the radioactivity in diacylglycerol moiety of PG.

Labeling		Chase	Percentage radioactivity								Total radioactivity in the lipid (kcpm)
°C	h	h	C-1 18:0 C-2 16:0	18:1 16:0	18:2 16:0	18:1 16:1	18:3 16:0	18:2 16:1	18:3 16:1	18:3 16:2	
38	0.1	--	80	20	0	0	0	0	0	0	22
38	1.0	--	23	71	6	0	0	0	0	0	239
38	1.0	10	2	50	48	0	0	0	0	0	243
38	Mass		(.5)	(56)	(41)	(0)	(0)	(0)	(0)	(0)	
22	0.1	--	65	35	0	0	0	0	0	0	7
22	1.0	--	11	60	24	0	5	0	0	0	108
22	1.0	10	0	4	22	0	74	0	0	0	132
22	Mass		(.3)	(10)	(26)	(0)	(61)	(0)	(0)	(0)	

acids esterified to PG. The radioactivity was confined to 16:0, 18:0 and 18:1 upon the labeling for 0.1 h at 38 and 22°C. After the labeling for 1.0 h, 16:0 and 18:1 were the most radioactive fatty acids at both 38 and 22°C. During the chase for 10 h, the radioactivity in 18:0 and 18:1 shifted to 18:2 at 38°C, and that in 18:1 and 18:2, to 18:3 at 22°C. On the other hand, the radioactivity in 16:0 scarcely shifted to 16:1 at both 38 and 22°C.

Table 11 shows distribution of the radioactivity among the molecular species of PG. The most radioactive species was 18:0/16:0 upon the labeling for 0.1 h at 38 and 22°C, and 18:1/16:0 was also labeled. After the labeling for 1.0 h, most of the radioactivity was found in 18:1/16:0, and a small amount was in 18:0/16:0 as well as 18:2/16:0. During the chase for 10 h, the radioactivity in 18:0/16:0 and 18:1/16:0 shifted to 18:2/16:0 at 38°C and to 18:3/16:0 at 22°C. These results were in good agreement with the changes in distribution of the radioactivity among the fatty acids (Table 10).

Sulfoquinovosyl diacylglycerol (SqDG)

Table 12 shows distribution of the radioactivity among the fatty acids esterified to SqDG. Upon the labeling for 0.1 h, 16:0 and 18:0 were most densely labeled at 38 and 22°C. After the labeling for 1.0 h, the most radioactive fatty acids were 16:0 and 18:0 at 38°C, and 16:0 and 18:1 at 22°C. During the chase for 10 h, the radioactivity shifted from 18:0 to 18:1 and 18:2 at 38°C, and from 18:0 and 18:1 to 18:2 and 18:3 at 22°C. The shift of radioactivity in the C_{18} acids, however, was slower in SqDG than in PG (Table 10) and GalDG(Table 6). Molecular species of SqDG were not studied because they were not efficiently

Table 12. Changes in distribution of the radioactivity among the fatty acids esterified to SqDG.

Labeling		Chase	Percentage radioactivity							Total radio-activity in fatty acids (kcpm)
°C	h	h	16:0	16:1	16:2	18:0	18:1	18:2	18:3	
38	0.1	--	52	0	0	40	6	1	0	4
38	1.0	--	53	0	0	31	15	1	0	106
38	1.0	10	55	1	0	4	27	12	1	130
38	Mass		(56)	(2)	(0)	(5)	(24)	(13)	(0)	
22	0.1	--	50	0	0	33	17	0	0	1
22	1.0	--	50	3	0	7	32	8	0	21
22	1.0	10	52	2	0	0	2	17	27	40
22	Mass		(53)	(4)	(0)	(1)	(5)	(8)	(29)	

resolved in the argentation TLC.

V. DISCUSSION

Based on the experimental results, we propose biosynthetic pathways of molecular species of monoglycolipids as presented in Fig. 6. Bold arrows indicate reactions in the main pathway and thin arrows indicate by-pass reactions. The primary product in the biosynthesis of glycolipids is the 18:0/16:0 molecular species of GlcDG. It is desaturated to 18:1/16:0 and 18:2/16:0 molecular species, and at 22°C, further to 18:3/16:0 species. In parallel with the desaturation, the 18:0/16:0, 18:1/16:0, 18:2/16:0 and 18:3/16:0 molecular species of GlcDG are converted to the corresponding molecular species of GalDG by epimerization of glucose to galactose (Chapter 2). The desaturation of 18:0/16:0 to 18:1/16:0 and 18:2/16:0, and at 22°C, to 18:3/16:0 takes place also in GalDG. The desaturation of the C_{16} acids occurs as well in GalDG. Thus, 18:1/16:1, 18:2/16:1 molecular species are formed at 38°C, while 18:2/16:1, 18:3/16:0, 18:3/16:1 and 18:3/16:2 molecular species are formed at 22°C.

The main pathways are determined according to the following consideration. We first compare the rates of the desaturation of 18:0 and the epimerization of glucose in 18:0/16:0-glucosylglycerol. Table 13 presents the distribution of radioactivity among 18:0/16:0 and 18:1/16:0 species of GlcDG and GalDG after the labeling for 0.1 h. The ratio of radioactivity in 18:1/16:0-glucosylglycerol to that in 18:0/16:0-galactosylglycerol is about 2.7 at 38°C and 12 at 22°C. It is concluded, therefore, that the desaturation of 18:0 and the epimerization of glucose in 18:0/16:0-glucosylglycerol take place at comparable

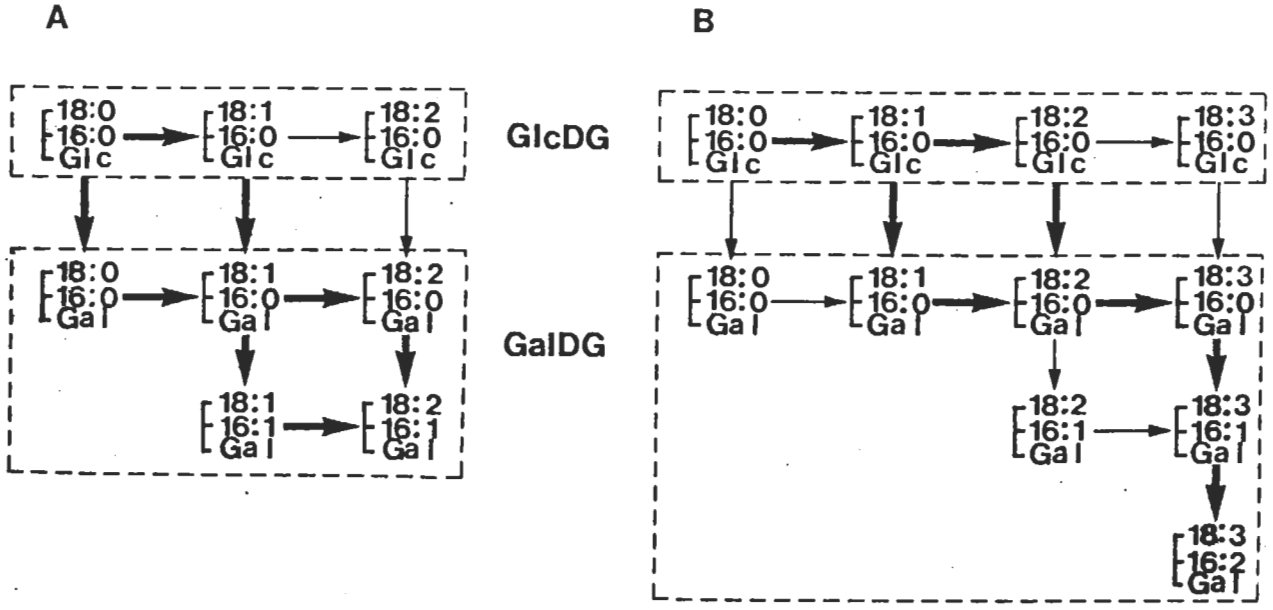

Fig. 6 Biosynthetic pathway of the molecular species of GlcDG and GalDG at 38 and 22°C. Glc, glucose; Gal, galactose. Reactions in the main pathway are illustrated by bold arrows and by-pass reactions are by thin arrows. A, 38°C; B, 22°C.

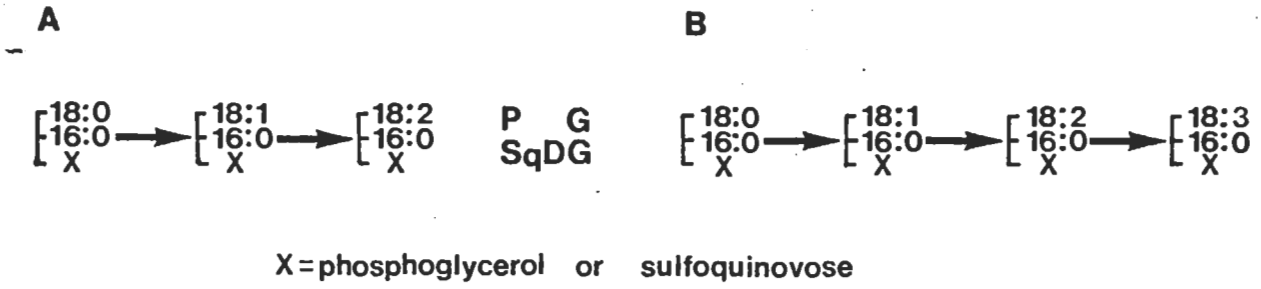

Fig. 7 Biosynthetic pathway of the molecular species of PG and SqDG at 38 and 22°C. X is phosphoglycerol in PG and sulfoquinovose in SqDG. A, 38°C; B, 22°C.

Table 13. Distribution of the radioactivity among the molecular species of GlcDG and GalDG after the labeling for 0.1 h. The values presented in kcpm are taken from Tables 5 and 7.

Lipid	Radioactivity in molecular species (kcpm)	
	18:0/16:0	18:1/16:0
At 38°C		
GlcDG	11.9	5.1
GalDG	1.9	1.5
At 22°C		
GlcDG	8.8	8.2
GalDG	0.7	0.5

Table 14. Distribution of the radioactivity among the molecular species of GlcDG and GalDG after the labeling for 1.0 h. The values presented in kcpm are taken from Tables 5 and 7.

Lipid	Radioactivity in molecular species (kcpm)			
	18:0/16:0	18:1/16:0	18:2/16:0	18:3/16:0
At 38°C				
GlcDG	20	148	34	0
GalDG	35	330	53	0
At 22°C				
GlcDG	20	92	48	5
GalDG	7	58	58	0

rates at 38°C, whereas the desaturation is much faster than the epimerization at 22°C.

Next, we compare the rates of the desaturation of 18:1 and the epimerization of glucose in 18:1/16:0-glucosylglycerol. Table 14 presents the distribution of radioactivity among the molecular species of GlcDG and GalDG after the labeling for 1.0 h. At 38°C, the radioactivity in 18:1/16:0-galactosylglycerol is ten times as high as that in 18:2/16:0-glucosylglycerol. At 22°C, on the other hand, the two molecular species are almost equally labeled. It is concluded, therefore, that the desaturation of 18:1 and the epimerization of glucose in 18:1/16:0-glucosylglycerol take place at comparable rates at 22°C, whereas the epimerization is much faster than the desaturation at 38°C.

The epimerization of glucose and the desaturation of 18:2 in 18:2/16:0-glucosylglycerol at 22°C can be compared in Table 14 in the same way. It is concluded that the epimerization is much faster than the desaturation.

The desaturation of fatty acids in GalDG depends on the growth temperature. The most distinct difference is found in the desaturation of 18:2, which is inactive at 38°C but very active at 22°C (Table 6). 16:1 is found in the 18:1/16:1 and 18:2/16:1 molecular species at 38°C, while it is principally in 18:3/16:1 molecular species at 22°C(Table 7). These findings indicate that the C_{18} and C_{16} acids are desaturated at comparable rates at 38°C, whereas the C_{18} acids are desaturated much faster than the C_{16} acids at 22°C. The desaturation of 16:0, in effect, occurs only after most of the C_{18} acids have been converted to 18:3 at 22°C.

It is noted that Gal_2DG and GalDG were appreciably different in the distribution of the radioactivity among the molecular species.

After the labeling for 1.0 h, the polyunsaturated molecular species of Gal_2DG (Table 9) were highly radioactive, whereas the corresponding molecular species of GalDG were scarcely labeled (Table 7). This difference does not mean an occurrence of an active desaturation of fatty acids in Gal_2DG. The insignificant shift of radioactivity among the polyunsaturated molecular species during the chase for 10 h (Table 9) rather suggests that the desaturation, if any, takes place only very slowly in this class of lipid. In this respect, it should be noticed that the distribution of radioactivity among the molecular species of Gal_2DG is similar to the mass distribution of GalDG, and that most of the radioactivity of the polyunsaturated molecular species is localized in the digalactosylglycerol moiety. All of these findings are consistent with the mechanism of biosynthesis of Gal_2DG (Chapter 2), in which the lipid is produced by transfer of a newly synthesized galactose unit to the pre-existing GalDG.

The pathway of biosynthesis of PG is illustrated in Fig. 7. The primary product is 18:0/16:0 molecular species. Stearic acid (18:0) at the C-1 position is sequentially desaturated to 18:1 and 18:2 at 38°C, and further to 18:3 at 22°C, whereas 16:0 at the C-2 position is not desaturated. The radioactivity in the molecular species of SqDG, though not determined, is estimated from the distribution of radioactivity among the fatty acids (Table 12). This is possible since the C_{18} and the C_{16} acids are esterified to the C-1 and the C-2 positions, respectively [8]. In this class of lipid, the primary product is also 18:0/16:0 species which is sequentially converted to 18:1/16:0, 18:2/16:0 at 38°C, and further to 18:3/16:0 at 22°C.

The finding that the 18:0/16:0 molecular species were primarily

synthesized and then sequentially desaturated to polyunsaturated molecular species suggests a possibility that the acyl groups remain esterified to the lipid molecules when they are desaturated (direct desaturation). Although the experimental results can also be accounted for by the deacylation-desaturation-reacylation, it is natural to assume that the complex lipids themselves are the substrates for the direct desaturation. A study using ^{13}C-labeling and mass spectrometry supports this view (Chapter 5). It is therefore reasonable to assume that every desaturation reaction in Eigs. 6 and 7 has its own kinetic parameter and regulation mechanism. This is verified by the fact that the desaturation of 18:1 and 16:0 in 18:1/16:0 of GalDG are modulated in different manners after a downward temperature shift (Chapter 4).

The difference in the biosynthetic pathways of lipid molecular species between A. variabilis and higher plants is now apparent. In this alga, the saturated fatty acids, 18:0 and 16:0, are primarily esterified to the lipids, and then desaturated. In higher plants, on the other hand, 18:0 is desaturated to 18:1 in the form of ACP thioester [54], and 18:1 is then incorporated to PC [51, 56] or GalDG [61, 63], and desaturated [51, 56, 61, 63-66]. In some plants containing 16:3 in GalDG, 16:0 is esterified to GalDG, and then desaturated [61, 63]. The involvement of PC in the desaturation of 18:1 [15, 46] and of GalDG in the desaturation of 18:1 and 16:0 [25] has been demonstrated in Chlorella vulgaris. These facts suggest that the mechanism for the desaturation of C_{18} acids is different between the blue-green alga and the photosynthetic eukaryotes, but that the mechanism for the desaturation of C_{16} acids seems similar among them.

Chapter 4 Effect of temperature shift on the biosynthesis of lipid molecular species

I. SUMMARY

Effect of downward temperature shift on the biosynthesis of lipid molecular species was studied by ^{14}C-labeling in Anabaena variabilis. After a temperature shift from 38 to 22°C, two types of accelerated desaturation of fatty acids were observed. The activities of conversion of 18:1/16:0 to 18:2/16:0, of 18:2/16:0 to 18:3/16:0, and of 18:2/16:1 to 18:3/16:1 in GalDG monotonously increased and attained a stationary level after 15 h. On the other hand, the activity of conversion of 18:1/16:0 to 18:1/16:1 in GalDG markedly increased for 3 h, then decreased with time and disappeared after 11 h. This transient increase in the activity of desaturation of 16:0 is the most rapid change in the lipid metabolism during the acclimation of the blue-green alga to the low temperature. The activities of conversion of 18:1/16:0 to 18:2/16:0 species in GlcDG, PG and SqDG and of 18:2/16:0 to 18:3/16:0 species in PG and SqDG also monotonously increased.

II. INTRODUCTION

The study in Chapter 3 indicates that the primary products of lipids in Anabaena variabilis are the 18:0/16:0 molecular species of GlcDG, SqDG and PG, which are converted to the unsaturated molecular species. The biosynthetic pathways of lipid molecular species are different between the cells which are isothermally grown at 38 and 22°C.

A temperature shift from 38 to 22°C induces a rapid and transient modification of the fatty acids and the lipid molecular species [28].

In order to elucidate the mechanism for this, we studied the conversion of lipid molecular species in GalDG and the desaturation of fatty acids in GlcDG, PG and SqDG with ^{14}C as a tracer.

III. MATERIALS AND METHODS

Culture conditions were the same as described in Chapter 2. For the temperature shift experiments, the algal cells which had been grown at 38°C for more than 10 days and were in a late logarithmic phase were inoculated into 30 volumes of fresh culture medium at 38°C. After the growth for 15 h, the culture flask was transferred to 22°C. The culture medium in the flask reached this temperature within 10 min.

When relative incorporation of ^{14}C into the lipid molecular species was studied, a portion of the culture was withdrawn before and after the temperature shift and incubated with $NaH^{14}CO_3$ (50 μCi/ml, 1 mM) for 1 h under illumination of incandescent lamp with an intensity of 6000 lux. The incubation temperature was 38°C before and 22°C after the temperature shift. Before the withdrawal of the portion, the culture was bubbled with air for 30 min to reduce the amount of dissolved CO_2. After the labeling with ^{14}C, the cells were subjected to analysis of the radioactivity in fatty acids and lipid molecular species.

When the photosynthetic incorporation of carbon atoms from bicarbonate into the lipid classes was determined, the concentration of dissolved CO_2 in the culture medium had to be determined. For this purpose, a portion of the culture was incubated with $NaH^{14}CO_3$ (2 μCi/ml, 0.04 mM) in the presence of added non-radioactive $NaHCO_3$ amounting to 3 and 10 mM. By comparing the radioactivities incorporated into the algal cells, the CO_2 concentration was determined to be about 3 mM at 22°C and 1 mM at

38°C.

In the study on the synthesis of 18:3/16:0 and 18:3/16:1 molecular species, the cells grown at 38°C were labeled with $NaH^{14}CO_3$ for 1 h. After washed with the culture medium, they were incubated for 10 h at 38°C as described in Chapter 2. At the end of this incubation, most of the radioactivity was accumulated in the 18:2/16:0 and 18:2/16:1 species of GalDG, and 18:1/16:0 and 18:2/16:0 species of PG and SqDG. The cells were further incubated at 22°C for 8 h, during which time a portion of the cells was withdrawn every 2 h for the analysis of radioactivity.

The lipids were extracted from the labeled cells and fractionated into lipid classes by TLC as described in Chapter 2. Monogalactosyl diacylglycerol (GalDG) was further separated into molecular species by argentation TLC. The radioactivity in the separated molecular species was measured by liquid scintillation counting. Monoglucosyl diacylglycerol (GlcDG), PG and SqDG were methanolyzed and the resultant fatty acid methyl esters were analyzed by radio-GLC. The radioactivity in Gal_2DG was not analyzed. Analytical procedures were the same as described in Chapters 2 and 3.

Chlorophyll a was extracted from the cells with 90 % methanol, and spectrophotometrically determined [171].

IV. RESULTS

Changes in incorporation of ^{14}C into lipid molecular species and fatty acids

Figure 8 shows changes in incorporation of the radioactivity into

the molecular species of GalDG after the temperature shift from 38 to 22°C. The relative incorporation of ^{14}C into 18:0/16:0 slightly decreased with time at 22°C. The relative incorporation of ^{14}C into 18:1/16:0 continuously decreased with time after the temperature shift and attained a stationary level after 15 h. On the contrary, the relative incorporation of ^{14}C into 18:2/16:0 continuously increased with time at 22°C for 15 h. The relative incorporation into 18:3/16:0, which was at the zero level before the temperature shift, gradually increased. On the other hand, the relative incorporation of ^{14}C into 18:1/16:1 markedly increased for 3 h and then decreased. No radioactivity was incorporated into 18:1/16:1 eleven hours after the temperature shift. The incorporation into 18:2/16:1 increased for 3 h and remained almost constant afterwards. The amounts of carbon atoms incorporated into the lipid classes were compared (Table 15). The photosynthetic incorporation of carbon atoms into GalDG increased three times in 9 h after the temperature shift.

These results suggest that the syntheses of 18:2/16:0 and 18:3/16:0 molecular species of GalDG are accelerated during the acclimation of the algal cells to the low temperature. The unique time course of the labeling of 18:1/16:1 suggests that the synthesis of this molecular species is once accelerated after the temperature shift but suppressed afterwards.

Figure 9 shows changes in incorporation of the radioactivity into the fatty acids of GlcDG, PG and SqDG after the temperature shift from 38 to 22°C. In GlcDG, the relative incorporation of the radioactivity into 18:0 decreased and that into 18:2 increased during the first 5 h after the temperature shift. They remained almost constant afterwards.

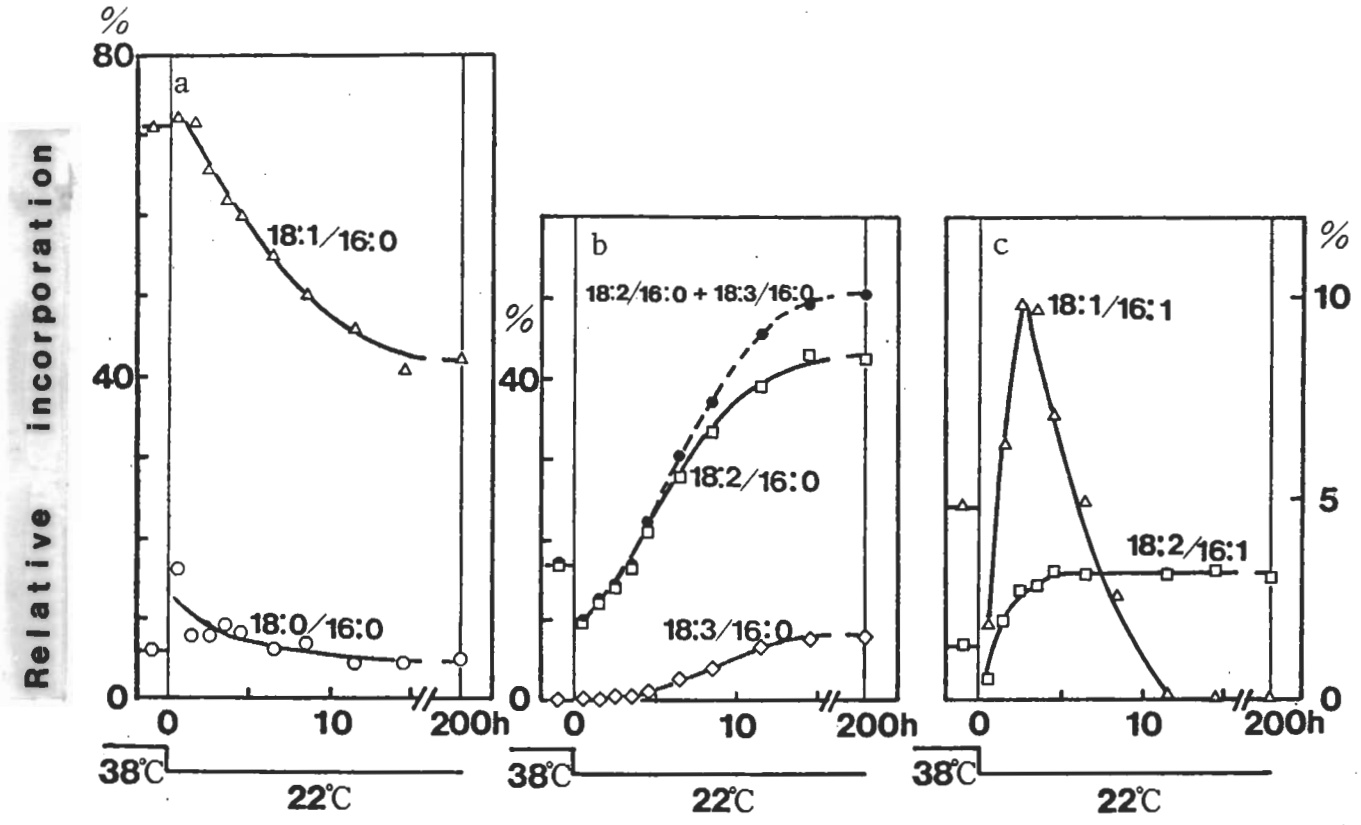

Fig. 8 Changes in incorporation of the radioactivity into the molecular species of GalDG before and after the temperature shift. A portion was withdrawn from the culture and the algal cells were incubated with $NaH^{14}CO_3$ for 1h. The points in the figure are at the center of the labeling time.
a, 18:0/16:0 and 18:1/16:0 species; b, 18:2/16:0 and 18:3/16:0 species; c, 18:1/16:1 and 18:2/16:1 species.

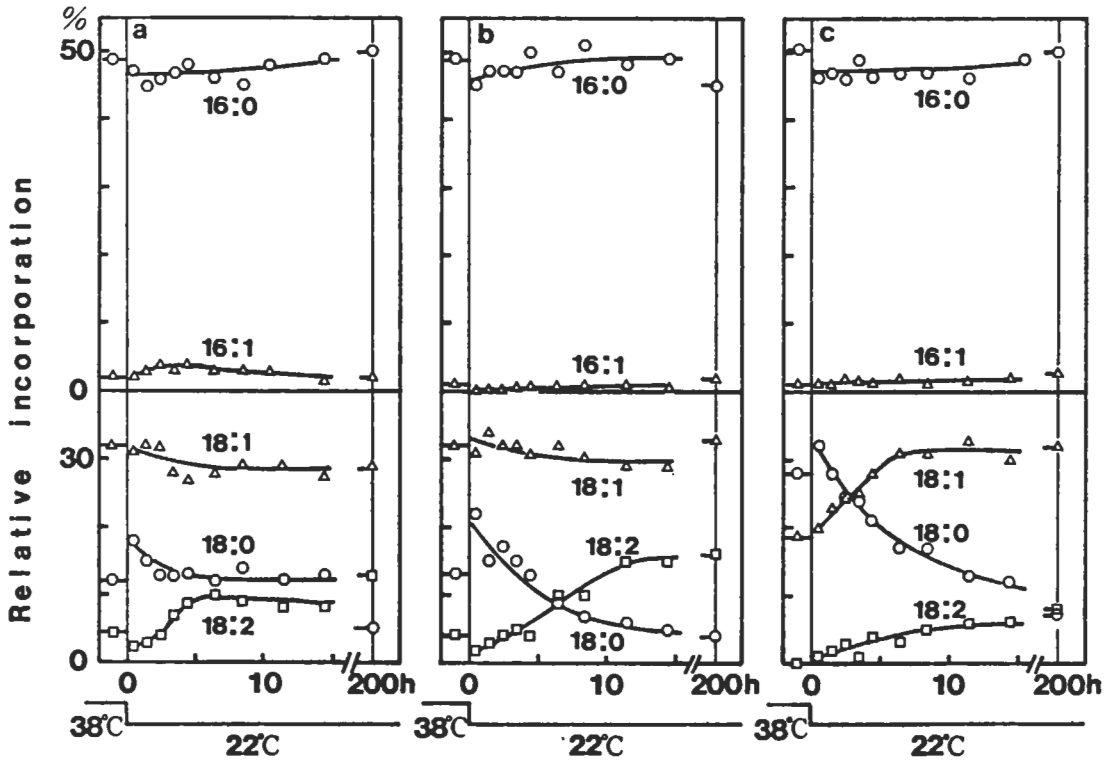

Fig. 9. Changes in incorporation of the radioactivity into the fatty acids of GlcDG, PG and SqDG before and after the temperature shift. The labeling procedure was the same as in Fig. 8.

a, GlcDG; b, PG; c, SqDG.

Table 15. Photosynthetic incorporation of carbon atoms into the lipid classes. The cells were labeled with $NaH^{14}CO_3$ for 1 h before and after the temperature shift from 38 to 22°C. The lipids were extracted immediately after the labeling.

Labeling time (h)*		Temperature of labeling (°C)	Incorporated carbon (μmol C/mg chl/h)					
From	To		GlcDG	GalDG	Gal_2DG	SqDG	PG	Total
Before temperature shift		38	3.6	7.8	0.8	2.5	4.4	19.1
0	1	22	1.0	1.0	0.2	0.5	2.5	5.2
4	5	22	1.6	1.8	0.5	0.9	2.7	7.6
8	9	22	1.3	2.7	0.7	0.8	3.4	8.9

* Time after the temperature shift.

The relative incorporation of ^{14}C into 18:1 and 16:0 remained almost unchanged. In PG, the relative incorporation of ^{14}C into 18:0 decreased and that into 18:2 increased continuously for 15 h. The relative incorporation of ^{14}C into 18:1 remained almost unchanged. In SqDG, the relative incorporation of ^{14}C into 18:0 decreased continuously for 15 h at 22°C, whereas that into 18:1 and 18:2 increased. The amounts of carbon atoms incorporated into GlcDG, PG and SqDG remained rather constant after the temperature shift (Table 15).

These results suggest that the desaturation reactions of the C_{18} acids in GlcDG, PG and SqDG are gradually accelerated during the acclimation of the algal cells to the low temperature.

Synthesis of polyunsaturated molecular species after the temperature shift

In the study on the synthesis of 18:3/16:0 and 18:3/16:1 molecular species of GalDG, temperature was shifted from 38°C to 22°C after most of the radioactivity had been accumulated in 18:2/16:0 and 18:2/16:1 species by the pulse-labeling and chase at 38°C. Figure 10 shows changes in distribution of the radioactivity among the molecular species of GalDG during the chase at 22°C. The relative radioactivity in 18:1/16:0 and 18:2/16:0 species decreased and that in 18:3/16:0 and 18:3/16:1 species increased. The relative radioactivity in 18:2/16:1 increased during the first 4 h and decreased afterwards. The total radioactivity in GalDG decreased by 12 % during the chase for 8 h. These results indicate that the synthesis of 18:3/16:0 and 18:3/16:1 species of GalDG from the preexisting molecular species started after the temperature shift.

Figure 11 shows changes in distribution of the radioactivity

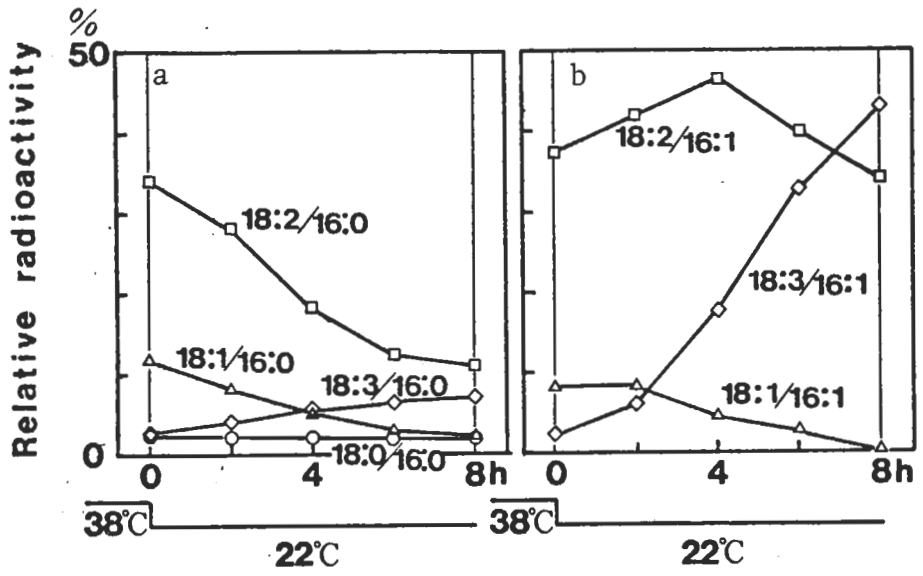

Fig. 10 Changes in distribution of the radioactivity among the molecular species of GalDG during the chase at 22°C. The cells were labeled with $NaH^{14}CO_3$ at 38°C for 1 h, incubated without ^{14}C at 38°C for 10 h and then at 22°C for 8 h.

a, 18:0/16:0, 18:1/16:0, 18:2/16:0 and 18:3/16:0 species;

b, 18:1/16:1, 18:2/16:1 and 18:3/16:1 species.

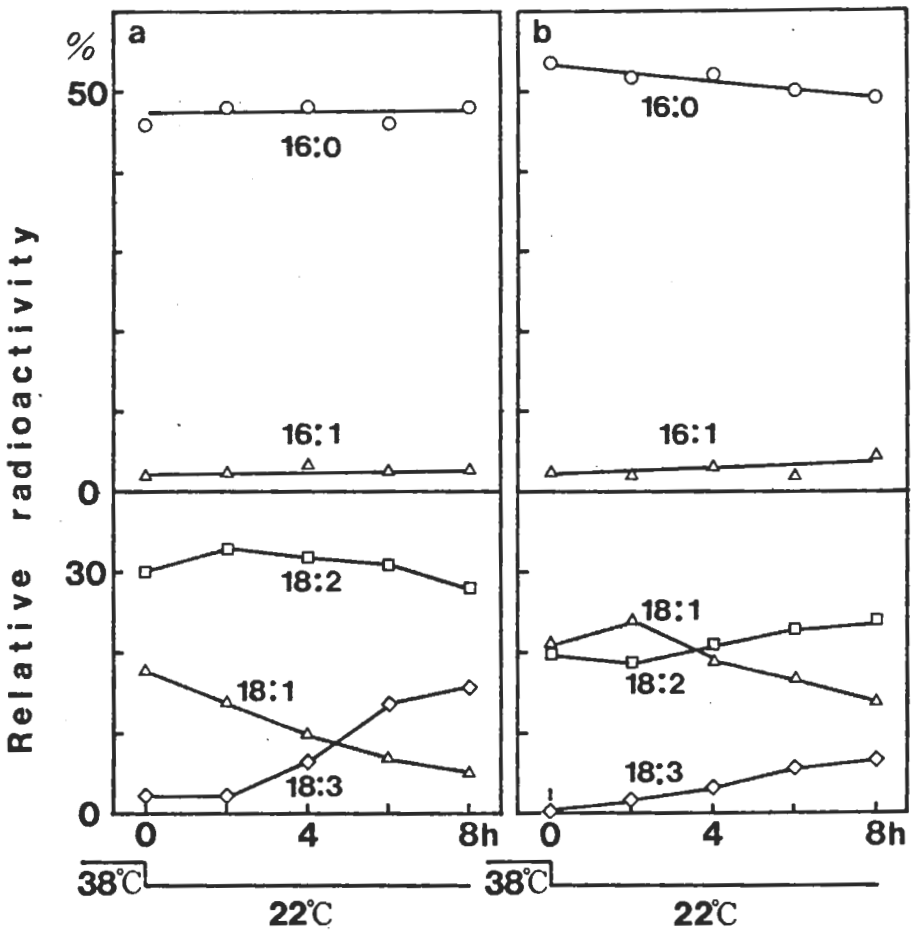

Fig. 11 Changes in distribution of the radioactivity among the fatty acids of PG and SqDG during the chase at 22°C. The procedures of labeling and chase were the same as in Fig. 10.

a, PG; b, SqDG.

among the fatty acids of PG and SqDG during the chase at 22°C. In PG, the relative radioactivity in 18:1 decreased and that in 18:3 increased. That in 18:2 slightly increased and then decreased. In SqDG, the relative radioactivity in 18:1 decreased and that in 18:2 and 18:3 increased. In both of the lipid classes, the relative radioactivity in 16:0 stayed at about 50 % during the chase at 22°C. The total radioactivity decreased by 18 % in PG but remained almost constant in SqDG during the chase for 8 h. Monoglucosyl diacylglycerol (GlcDG) was not studied in the pulse-labeling and chase experiment, since the radioactivity had already shifted to GalDG when the temperature was shifted to 22°C.

These results indicate that, in PG and SqDG, the synthesis of 18:3 from the pre-existing 18:1 and 18:2 started after the temperature shift.

V. DISCUSSION

In the biosynthesis of molecular species of GalDG in A. variabilis (Chapter 3), 18:1/16:0 is sequentially converted to 18:2/16:0 and 18:3/16:0 by the desaturation of the C_{18} acids at the C-1 position of glycerol moiety. The conversion of 18:1/16:0 to 18:1/16:1 by the desaturation of 16:0 at the C-2 position also takes place. The time course of the incorporation of the radioactivity into 18:1/16:1 (Fig. 8) can be regarded as an approximate measure of change in desaturation activity which converts 18:1/16:0 to 18:1/16:1. The time courses of the incorporation of the radioactivity into 18:3/16:0, and (18:2/16:0 + 18:3/16:0) (dashed line in Fig. 8) can be approximate measures of the changes in the desaturation activities which convert 18:2/16:0 to 18:3/16:0 and 18:1/16:0 to 18:2/16:0, respectively. For simplicity, the desaturation reactions which produce 18:2/16:1 are not involved in this consideration. This is

justified by the fact that the radioactivity found in 18:2/16:1 molecular species was very low (less than 3 % of the total radioactivity in GalDG).

Based on these findings, we can estimate that the activity of desaturation which converts 18:1/16:0 to 18:2/16:0 in GalDG increases with time and attains the stationary level after 15 h. On the other hand, the activity of desaturation which converts 18:1/16:0 to 18:1/16:1 increases for about 3 h, decreases afterwards and disappears after 11 h. The activity of desaturation which converts 18:2/16:0 to 18:3/16:0, which is not found just after the temperature shift, begins to increase at 5 h and attains the stationary level after 15 h.

The activity of desaturation which converts 18:1/16:0 to 18:2/16:0 in GlcDG, PG and SqDG can be estimated from the incorporation of radioactivity into 18:2 (Fig. 9). This is possible since the C_{18} acids are esterified to the C-1 position, and 16:0 is to the C-2 position of glycerol moiety in these lipid classes [8]. The activity of desaturation which converts 18:1/16:0 to 18:2/16:0 in GlcDG, PG and SqDG monotonously increases with time and attains the stationary level after 15 h.

The changes in the activities of desaturation can be classified into two types. The first type includes the desaturation of 18:1 to 18:2 in 18:1/16:0 species of GalDG, GlcDG, PG and SqDG (Figs. 8 and 9) and the desaturation of 18:2 to 18:3 in 18:2/16:0 species of GalDG (Fig. 8), PG and SqDG (Fig. 11). The activities of desaturation in this type monotonously increase with time after the temperature shift. The desaturation of 18:2 to 18:3 in 18:2/16:1 species of GalDG can be classified in this type, since the synthesis of 18:3/16:1 does not occur just after the temperature shift and increases afterwards (Fig. 10). The desaturation of 18:0 to 18:1 in 18:1/16:0 species of GlcDG, PG and SqDG can be

also of this type, since the relative incorporation into 18:0 gradually decreased during the growth at 22°C (Fig. 9).

The second type includes the desaturation of 16:0 to 16:1 in 18:1/16:0 of GalDG. The desaturation activity markedly increases for 3 h after the temperature shift, decreases afterwards and disappears after 11 h. This transient change in the desaturation activity at 22°C most efficiently reduces the amount of 18:1/16:0 species of GalDG during the acclimation of the algal cells to the low temperature.

The modification of biosynthetic pathway of lipid molecular species after the downward temperature shift is summarized in Fig. 12. Bold arrows indicate the desaturation reactions which are gradually accelerated after the temperature shift, whereas an open arrow indicates the desaturation reaction which is only transiently accelerated. The mode of regulation of the desaturation reactions which convert 18:0/16:0 to 18:1/16:0, 18:1/16:1 to 18:2/16:1, 18:2/16:0 to 18:2/16:1, 18:3/16:0 to 18:3/16:1, and 18:3/16:1 to 18:3/16:2 in GalDG and the epimerization of the molecular species of GlcDG to the corresponding species of GalDG can not be determined at present.

In the consideration of changes in the desaturation activities described above, the 18:2/16:0 species of GalDG is assumed to be synthesized solely from the 18:1/16:0 species. As studied in Chapter 3, however, a part of 18:2/16:0 of GalDG is synthesized from 18:2/16:0 of GlcDG at 22°C, and the incorporation of the radioactivity into (18:2/16:0 + 18:3/16:0) (Fig. 8b) may include the activity of epimerization which converts the 18:2/16:0 species of GlcDG to the 18:2/16:0 species of GalDG.

The increase in the activity of desaturation is likely to suggest that the increased synthesis of desaturation enzymes (desaturases) are

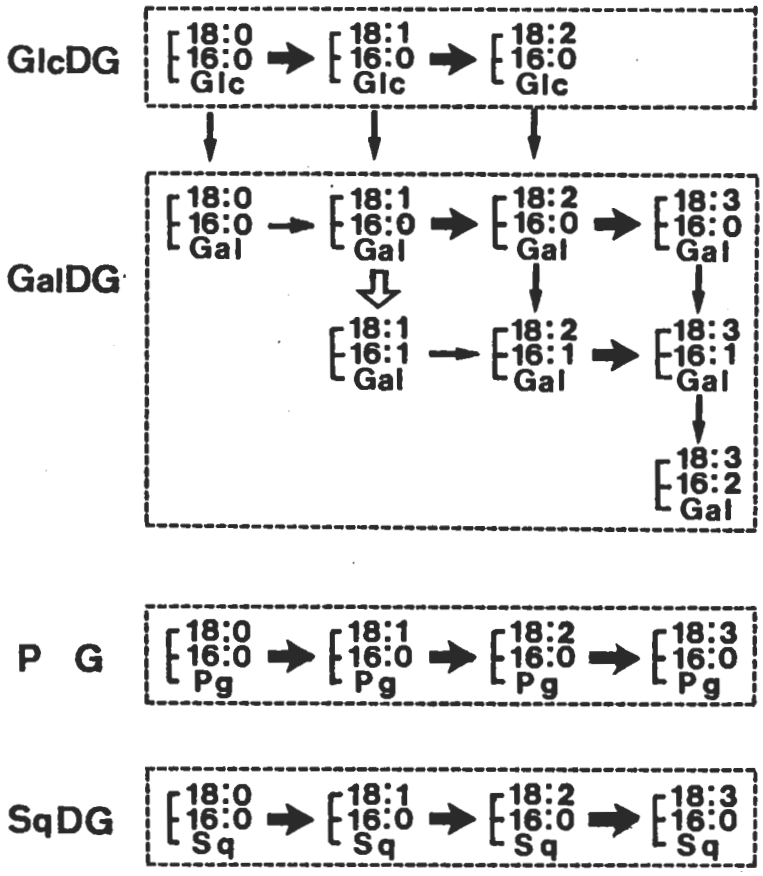

Fig. 12. A scheme for the modification of desaturation pathway of lipid molecular species after the temperature shift. Bold arrows indicate desaturation reactions which are monotonously accelerated. An open arrow indicates a desaturation reaction which is transiently accelerated. As to the reactions shown by thin arrows, the mode of changes in the activities after the temperature shift was not determined. Glc, glucose; Gal, galactose; Pg, phosphoglycerol; Sq, sulfoquinovose.

involved in the accelerated desaturation of fatty acids after the temperature shift. This inference is verified by the fact that chloramphenicol and rifampicin inhibit the temperature shift-induced desaturation of fatty acids in A. variabilis [170].

The transient increase in the activity of desaturation of 16:0 to 16:1 in 18:1/16:0 of GalDG appears similar to the "hyperinduction" of desaturases in Bacillus megaterium [125], in which the synthesis of the desaturases is rapidly induced at low temperatures and then partially suppressed by a repressor protein [127]. However, a notable difference exists between the "hyperinduction" in B. megaterium and the accelerated desaturation in A. variabilis. In B. megaterium, the activity of desaturation, which is not detected just after the downward temperature shift, increases with time and afterwards decreases to a stationary level. In A. variabilis, the activity of desaturation, which is present just after the temperature shift, increases with time, decreases afterwards and disappears. The difference may suggest that the activity of desaturation of 16:0 to 16:1 in A. variabilis is regulated by a mechanism different from the "hyperinduction" in B. megaterium.

Chapter 5 Evidence for the direct desaturation of palmitic acid esterified to monogalactosyl diacylglycerol

I. SUMMARY

The mechanism for the desaturation of 16:0 esterified to GalDG was studied in intact cells of Anabaena variabilis by "isotope combination analysis" which involved ^{13}C-labeling and mass spectrometry. Cells grown at 38°C were fed with $Na_2{}^{13}CO_3$ at 38°C for 2.5 h and then incubated at 38 and 22°C for 7.5 h in the presence of cerulenin. GalDG was isolated from the algal cells before and after the incubation, and the acyl group at the C-1 position was removed by treating the lipid with a lipase from Rhizopus delemar. The resultant 2-acyl-3-galactosyl-sn-glycerol was trimethylsilylated and analyzed by mass spectrometry.

The experimental results indicated that GalDG isolated before the incubation contained molecules whose glycerol and 16:0 moieties were both enriched with ^{13}C (enriched-enriched combination) and molecules not enriched with ^{13}C (unenriched-unenriched combination), but practically no hybrid molecules in which either glycerol or 16:0 moiety was enriched with ^{13}C (enriched-unenriched combinations). The 16:1-containing species was little enriched with ^{13}C. After the incubation for 7.5 h, during which time 16:0 at the C-2 position was desaturated to 16:1, the 16:1-containing species was composed of the enriched-enriched and unenriched-unenriched combinations but little enriched-unenriched combinations.

Based on these findings, we conclude that 16:0 esterified to GalDG is directly desaturated in vivo. The accelerated conversion of 16:0 to 16:1 after a downward temperature shift was also the direct desaturation.

II. INTRODUCTION

Several mechanisms have been proposed for the synthesis of unsaturated fatty acids in the complex lipids. In Escherichia coli, a double bond is introduced into the hydrocarbon chain during the synthesis of fatty acids in the "anaerobic pathway" [107]. In Tetrahymena [145], 16:0 is desaturated to 16:1 in the form of acyl-CoA. 18:0 is desaturated to 18:1 in the form of acyl-ACP in higher plants [54] and in the form of acyl-CoA in animals [167]. The desaturation of 18:1 to 18:2 after the fatty acid is esterified to PC has been demonstrated in Chlorella [44,46], Neurospora [155], yeasts [151,154] and higher plants [64,65].

The results in tracer experiments with $NaH^{14}CO_3$ (Chapters 2 and 3) show that, in Anabaena variabilis, ^{14}C-16:0 is esterified to GalDG and then desaturated to ^{14}C-16:1. This finding has made us to consider a mechanism in which the C_{16} acid remains esterified to the galactolipid when it is desaturated. This mechanism is termed "direct desaturation". Another possible mechanism involves a deacylation-desaturation-reacylation cycle. In this case, 16:0 is desaturated in the thioester form as in the desaturation of 16:0 in Tetrahymena [145] and of 18:0 in higher plants [54] and animals [167]. In order to determine which mechanism would be operative in A. variabilis, we developed "isotope combination analysis" which involved ^{13}C-labeling of the intact cells and mass spectrometric analysis of lipids. The desaturation of 16:0 in GalDG after the temperature shift from 38 to 22°C was studied as well.

III. THEORETICAL

Basic ideas

The study in Chapter 3 indicates that 18:1/16:0 is highly labeled

among the molecular species of GalDG after the labeling with $NaH^{14}CO_3$ for 1 h at 38°C, and that 18:1 and 16:0 are desaturated during the subsequent chase. The desaturation of 16:0 to 16:1 in GalDG illustrated in Fig.13A can be explained by either the direct desaturation or the deacylation-desaturation-reacylation mechanism.

These two mechanisms can be distinguished, when the C_{18}/16:0 species include unlabeled and labeled molecules as shown in Figs. 13B and C (this situation is established under the experimental condition employed in the study of this chapter, see RESULTS). The direct desaturation should produce only unlabeled and labeled C_{18}/16:1 species of GalDG (Fig. 13B). In the deacylation-desaturation-reacylation mechanism (Fig. 13C), the labeled GalDG is hydrolyzed to labeled 16:0 and labeled lyso-GalDG, and the unlabeled GalDG is hydrolyzed to unlabeled 16:0 and unlabeled lyso-GalDG. After the desaturation of 16:0 to 16:1, the labeled and unlabeled 16:1's are re-esterified either to the labeled or to the unlabeled lyso-GalDG. As a result, partially labeled hybrid species of C_{18}/16:1 are to be produced in the deacylation-desaturation-reacylation mechanism. Whether the hybrid molecules are produced or not will answer which mechanism operates in the desaturation of 16:0 in GalDG.

The mechanism of direct desaturation illustrated in Fig. 13B, however, includes a case of tightly coupled deacylation-desaturation-reacylation, in which a deacylation product, fatty acid, does not separate from the other product, lyso-GalDG, and, after the desaturation, recombines with the same molecule of lyso-GalDG as before. This is possible, for example, in a case where both the fatty acid and lyso-GalDG are kept attached to the desaturation enzyme as an enzyme-substrate complex during the reactions of deacylation, desaturation and reacylation. Though this case is a little

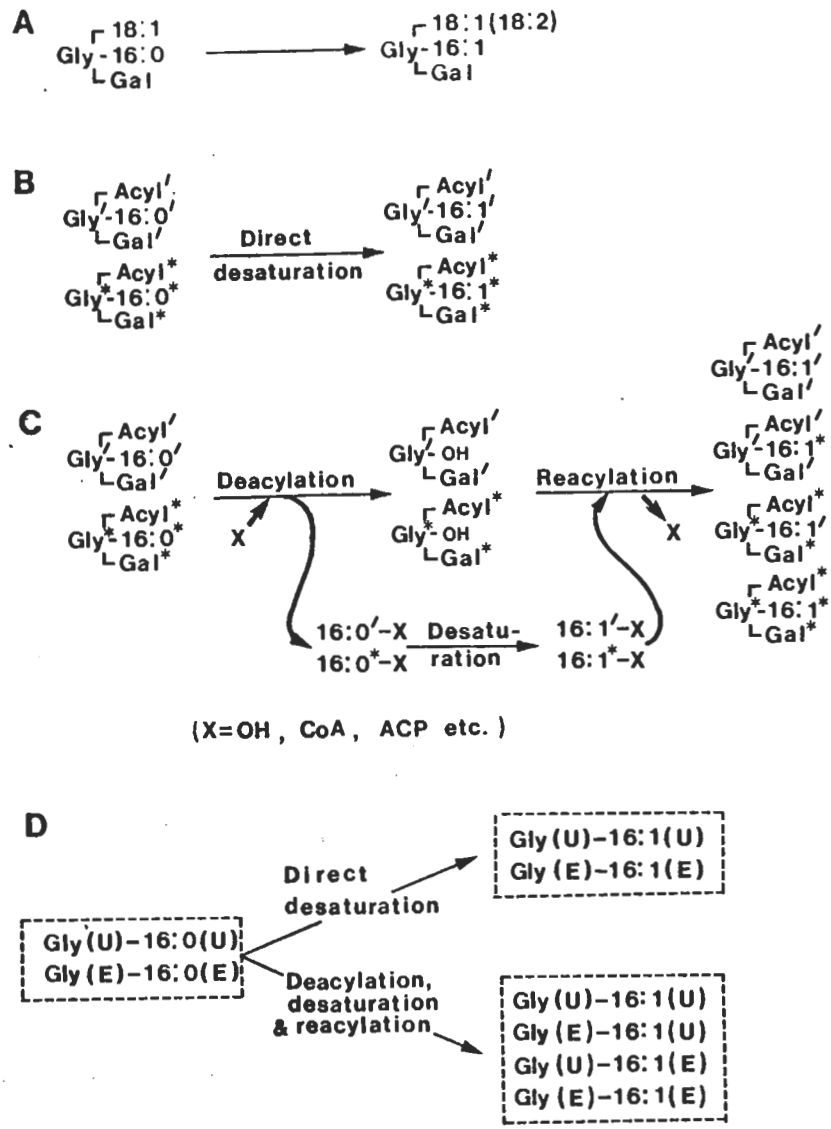

Fig.13. Schemes for the changes of the isotope combinations during the desaturation of 16:0 in GalDG.

A, Desaturation of 16:0 in 18:1/16:0 molecular species of GalDG.

B, Desaturation of 16:0 in labeled and unlabeled GalDG by the direct desaturation. Labeling and unlabeling are indicated by asterisks and apostrophes, respectively. C, Desaturation of 16:0 in labeled and unlabeled GalDG by the deacylation-desaturation-reacylation mechanism. Symbols are the same as in B. D, Principle of the present study. The combination of the glycerol backbone and the acyl group at the C-2 position in GalDG is indicated as Gly-16:0 and Gly-16:1. (U), unenriched; (E), enriched.

different from the direct desaturation in a strict sense, both mechanisms can not be discriminated by the method employed here.

Amounts of the hybrid molecular species cannot be determined by tracing the radioactive isotopes. Instead, a method involving ^{13}C-labeling and mass spectrometry was employed. In this potent technique, the amounts of the molecular species containing different numbers of ^{13}C atoms can be determined from the mass spectra.

Plan of experiment

In the study on the mechanism of desaturation of 16:0 at the C-2 position of GalDG, it is necessary to remove the acyl group at the C-1 position and the galactose unit at the C-3 position. The acyl group at the C-1 position can be eliminated by selective hydrolysis with a lipase from Rhizopus delemar. The galactose unit at the C-3 position is removed upon fragmentation by electronic ionization in the mass spectrometric measurement (see RESULTS). Thus, a fragment which contains a glycerol backbone and an acyl group at the C-2 position is obtained, and its mass spectrum can be measured.

Definition of terms

In the followings, a term "to enrich a substance with ^{13}C" is used instead of "to label a substance with ^{13}C", in order to avoid a possible confusion arising from the fact that even unlabeled molecules also contain ^{13}C on a natural abundance level(1.107 %). "To enrich a substance with ^{13}C" means "to increase the content of ^{13}C in a substance above the natural abundance level". It should be mentioned that "enriched" and "unenriched" are used to qualify a group of molecules but not a single molecule. In this respect, it is useful to consider, for example, a group of enriched molecules of fatty acids having 16 carbon atoms.

When ^{12}C and ^{13}C both amount to 50 % in this group, the content of ^{13}C atoms in the molecule shows a binomial distribution with a maximum at eight atoms of ^{13}C. Unenriched molecules are expressed by "(U)" and molecules enriched with ^{13}C are by "(E)". A combination of enriched or unenriched glycerol, and enriched or unenriched 16:0 (or 16:1) is termed "isotope combination", and the four possible combinations are expressed as U-U, E-U, U-E and E-E.

Principle

The principle of the "isotope combination analysis" is illustrated in Fig. 13D. By the direct desaturation (Fig. 13B), Gly(U)-16:0(U) and Gly(E)-16:0(E) are converted to Gly(U)-16:1(U) and Gly(E)-16:1(E). By the deacylation-desaturation-reacylation mechanism, on the other hand, the hybrid molecules, Gly(E)-16:1(U) and Gly(U)-16:1(E) are produced in addition to Gly(U)-16:1(U) and Gly(E)-16:1(E).

Determination of the relative amounts of isotope combinations

A qualitative estimation of the possible mechanism of the fatty acid desaturation can be achieved according to the amounts of the E-U and U-E combinations of Gly-16:1 produced by the desaturation. If Gly(E) and C_{16}(E) contained three and sixteen ^{13}C atoms, respectively, but no other isotopes and if Gly(U) and C_{16}(U) contained no ^{13}C atoms, Gly(E)-C_{16}(E), Gly(U)-C_{16}(E) and Gly(E)-C_{16}(U) should be heavier than Gly(U)-C_{16}(U) by 19, 16 and 3 mass units, respectively. In this case, the relative amounts of the isotope combinations can be directly determined from the mass spectrum.

In the experiments, however, this situation is not established owing to the following reasons. Firstly, the stable isotopes of ^{2}H, ^{17}O, ^{18}O, ^{29}Si and ^{30}Si of natural abundance distort the mass spectrum. Secondly,

the unenriched molecules contain a significant level (1.107 %) of ^{13}C atoms. Thirdly, the ^{13}C-content in the enriched molecules and fragments is about 50 % in average and, thus, each of the E-U, U-E and E-E combinations contains molecules having a wide variety of molecular weights. As a result, the relative amounts of the isotope combinations cannot be determined directly from the mass spectrum of the fragment. Therefore, the relative amounts of isotope combinations are determined by the following calculation involving three steps.

In the first step, the observed mass spectra of TMS-Gly-16:0 and TMS-Gly-16:1 are converted to the ^{13}C-content distributions of Gly-16:0 and Gly-16:1 by a mathematical treatment according to Appendix 1. The observed mass spectra of methyl esters of 16:0 and 16:1, and tris(TMS)-glycerol are converted to the ^{13}C-content distributions of 16:0, 16:1 and glycerol in similar ways. On a simple and reasonable assumption, the ^{13}C-content distributions of 16:0(U), 16:0(E), 16:1(U), 16:1(E), Gly(U) and Gly(E) are estimated from the ^{13}C-content distributions of 16:0, 16:1 and Gly (see RESULTS).

In the second step, the ^{13}C-content distributions of Gly(U), Gly(E), 16:0(U), 16:0(E), 16:1(U) and 16:1(E) are used to calculate the ^{13}C-content distributions of U-U, E-U, U-E and E-E combinations of Gly-16:0 and Gly-16:1 by mathematical convolution according to Appendix 2.

The observed ^{13}C-content distributions of Gly-16:0 and Gly-16:1 are to be a linear combination of the ^{13}C-content distributions of U-U, E-U, U-E and E-E. In the third step, therefore, the relative amounts of the four isotope combinations in Gly-16:0 and Gly-16:1 are estimated by simulating the observed ^{13}C-content distribution by linear combination of the ^{13}C-content distributions of the four isotope combinations which

Table 16. A theoretical estimation of the relative amounts of isotope combinations of Gly-16:0 and Gly-16:1 after the desaturation.

	Before desaturation		After desaturation			
	Gly-16:0	Gly-16:1	Gly-16:0	Gly-16:1		
				Direct desaturation	Deacylation-desaturation-reacylation	Direct desaturation(1-d) and deacylation-desaturation-reacylation(d)
U-U	A(1-x-y-z)	B(1-u-v-w)	(A-C)(1-x-y-z)	B(1-u-v-w)+ C(1-x-y-z)	B(1-u-v-w)+ C(1-y-z)(1-x-z)	B(1-u-v-w)+ (1-d)C(1-x-y-z)+ dC(1-y-z)(1-x-z)
E-U	Ax	Bu	(A-C)x	Bu+Cx	Bu+C(1-y-z)(x+z)	Bu+(1-d)Cx+dC(1-y-z)(x+z)
U-E	Ay	Bv	(A-C)y	Bv+Cy	Bv+C(y+z)(1-x-z)	Bv+(1-d)Cy+dC(y+z)(1-x-z)
E-E	Az	Bw	(A-C)z	Bw+Cz	Bw+C(y+z)(x+z)	Bw+(1-d)Cz+dC(y+z)(x+z)
Total	A	B	A-C	B+C	B+C	B+C

are calculated in the second step.

Estimation of the contribution of the two mechanisms

The contributions of the two mechanisms in the desaturation of 16:0 is estimated quantitatively by using parameters as illustrated in Table 16. The total amounts of Gly-16:0 and Gly-16:1 before the desaturation are A and B moles, respectively. Relative amounts of U-U, E-U, U-E and E-E combinations are (1-x-y-z), x, y, z in Gly-16:0 and (1-u-v-w), u, v, w in Gly-16:1. Though the values for x, y, u, v and w are to be zero in the ideal case as shown in Fig. 13D, in the experiments they are distinct, but very small if compared to the value for z.

The relative amounts of U-U, E-U, U-E and E-E combinations in Gly-16:0 and Gly-16:1 after the desaturation of C moles of Gly-16:0 can be calculated from these values as in the followings. In case of the direct desaturation, C(1-x-y-z) moles of U-U, Cx moles of E-U, Cy moles of U-E and Cz moles of E-E of Gly-16:1 should be produced (Column 4 of Table 16). In the deacylation-desaturation-reacylation mechanism, C(1-y-z) moles of 16:0(U) and C(y+z) moles of 16:0(E) should be released, desaturated, and recombined with C(1-x-z) moles of Gly(U) and C(x+z) moles of Gly(E). The amounts of the produced U-U, E-U, U-E and E-E combinations of Gly-16:1 are listed in Column 5 of Table 16.

When both of the desaturation mechanisms concurrently operate, the relative amounts of isotope combinations are calculated by using the relative contribution of the deacylation-desaturation-reacylation mechanism, d. They are listed in Column 6 of Table 16. Here a summed amount of E-U and U-E of Gly-16:1, h, is calculated:

$$h = \frac{[Bu + (1-d)Cx + dC(1-y-z)(x+z)] + [Bv + (1-d)Cy + dC(y+z)(1-x-z)]}{B + C} \qquad \text{----------(1)}$$

From Eq.(1), d is calculated:

$$d = \frac{(B/C)(h-u-v) + (h-x-y)}{2[z - (y+z)(x+z)]} \qquad \text{----------(2)}$$

From the mass spectra, the relative amounts of U-U, E-U, U-E and E-E in Columns 1, 2, 3 and 6 of Table 16 are estimated as described above. Therefore, the values for x, y, z, u, v and h are obtained. The ratio, (B/C), is determined in a separate measurement (see RESULTS). Thus, the relative contributions of the deacylation-desaturation-reacylation (d) and the direct desaturation (1-d) can be estimated.

IV. MATERIALS AND METHODS

A. variabilis was grown photoautotrophically at 38°C in Kratz and Myers' medium C [158] with aeration by 1 % CO_2/air as described in Chapter 2.

A culture (500 ml) in the middle-logarithmic phase was used for experiments. By aerating the culture with air in the light (6000 lux) for 0.5 h, the concentration of inorganic carbon was reduced to about 2 mM. Then, 600 mg $Na_2{}^{13}CO_3$ (90 % ^{13}C, final concentration 9.3 mM) dissolved in 100 ml fresh culture medium was added. The culture was incubated at 38°C in the light (6000 lux) with continuous stirring for 2.5 h under nitrogen atmosphere. In order to terminate the incorporation of ^{13}C into lipids, 6 mg cerulenin dissolved in 0.5 ml ethanol was added (final concentration, 10 μg/ml). After incubation for 10 min, the culture was divided into three portions: the first portion was immediately subjected to lipid extraction ("before incubation"). The second and the third portions were incubated in the light at 38 and 22°C, respectively, with

continuous bubbling by 1 % CO_2/air for 7.5 h. The light intensity was 6000 lux for the incubation at 38°C and 3000 lux for the incubation at 22°C. Then, these portions were subjected to lipid extraction ("after incubation").

Experimental procedures before analysis by gas chromatography-mass spectrometry are summarized in Fig. 14. Lipids were extracted with $CHCl_3$/CH_3OH (2:1, v/v) from the cells which were collected by centrifugation at 1000 x g for 5 min. Monogalactosyl diacylglycerol (GalDG) was isolated by TLC on silica gel (Merck 5721, precoated plates) by using acetone/benzene/water (91:30:8, by vol.) [159] as developing solvent. This lipid was enzymatically hydrolyzed with a lipase (EC 3.1.1.3) from Rhizopus delemar [8] which liberates the fatty acids from the C-1 position. The resultant 2-palmitoyl- and 2-palmitoleoyl-galactosylglycerols were separated by TLC on $AgNO_3$-impregnated silica gel plates prepared as described in Chapter 3. The same solvent system as above was used for development. Cross contamination of the 16:0- and 16:1-species, which interfered with the mass spectra, was reduced to less than 0.5 % by repeated argentation TLC.

A part of separated 2-acyl-galactosylglycerol was trimethylsilylated by a mixture of hexamethyldisilazane and trimethylchlorosilane in pyridine. The resultant 1-TMS-2-acyl-3-tetrakis(TMS)galactosylglycerol was analyzed by gas chromatography-mass spectrometry equipped with a glass column (0.5 m x 3 mm i.d.) packed with 1.5 % OV-1 on Chromosorb W (acid washed and DMCS-treated) run at 250°C. The flow rate of He carrier gas was 40 ml/min. The mass distribution of a fragment ion having one TMS group, one acyl group and one glycerol backbone was recorded.

The other part of separated 2-acyl-galactosylglycerol was methanolyzed

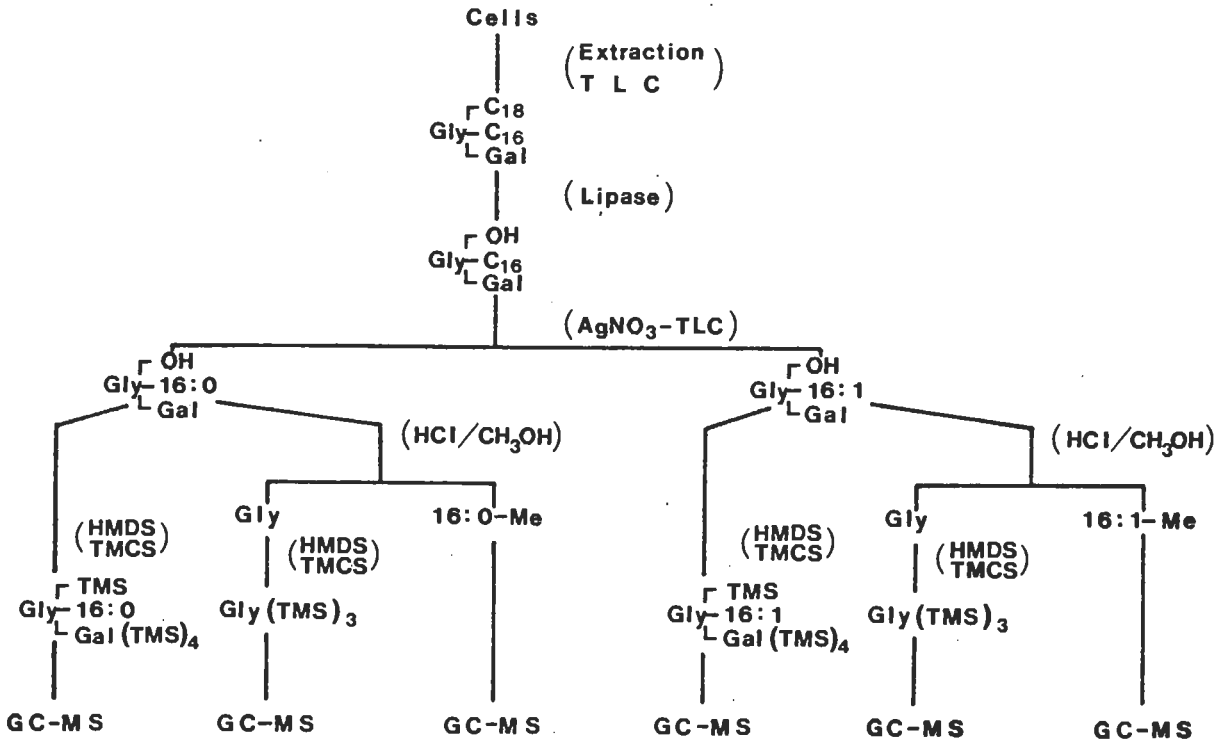

Fig. 14 Experimental procedures for the treatment of GalDG prior to the gas chromatography-mass spectrometry analysis. Methyl galactoside, which is produced by the methanolysis, was not analyzed by gas chromatography-mass spectrometry. HMDS, hexamethyldisilazane; TMCS, trimethylchlorosilane; GC-MS, gas chromatography-mass spectrometry.

in 3 % HCl/CH_3OH at 85°C for 2.5 h. Fatty acid methyl esters were extracted with n-hexane, whereas glycerol and methyl galactoside were recovered from the methanol phase.

The fatty acid methyl esters were directly analyzed by gas chromatography-mass spectrometry equipped with a glass column (2 m x 3 mm i.d.) packed with 2 % diethyleneglycol adipate on Chromosorb W (acid washed and DMCS-treated) run at 170°C. The flow rate of He carrier gas was 40 ml/min. The mass distribution of molecular ion, M^+, of methyl palmitate and those of $(M-31)^+$ and $(M-32)^+$ ions of methyl palmitoleate which contain acyl group were recorded.

Glycerol and methyl galactoside were trimethylsilylated with a mixture of hexamethyldisilazane and trimethylchlorosilane in pyridine and the resultant tris(TMS)glycerol and methyl tetrakis(TMS)galactoside were analyzed by gas chromatography-mass spectrometry equipped with a glass column (2 m x 3 mm i.d.) packed with 1.5 % OV-1 on Chromosorb W (acid washed and DMCS-treated). The column temperature was first 140°C for 2 min, and after tris(TMS)glycerol had been eluted the temperature was raised to 220°C to elute out methyl tetrakis(TMS)galactoside. The mass distributions of $(M-90)^+$ and $(M-91)^+$ ions of tris(TMS)glycerol containing two TMS groups and one glycerol backbone were recorded. Methyl tetrakis-(TMS)galactoside was not examined by mass spectrometry.

Ionization condition used in the mass spectrometry was as follows: The instrument used was Shimadzu-LKB 9000B. The ionizing current, 60 μA. The ionization voltage, 20 eV. The ion source temperature, 270°C.

The retention times in gas chromatography of the ^{12}C-compounds and the highly ^{13}C-enriched compounds were almost identical. Several mass spectra were taken near the top of the peak, averaged and used for the

isotope combination analysis.

All the calculations were performed with a microcomputer (PC-8001, Nippon Electric Co., Tokyo).

$Na_2{}^{13}CO_3$ was a product of Prochem(London, U.K.). Cerulenin was from Makor Co. (Jerusalem, Israel). Rhizopus lipase was from Seikagaku Kogyo Co. (Tokyo, Japan). The trimethylsilylating reagents were from Tokyo Chemical Industry (Tokyo, Japan). Other chemicals and solvents were from Wako Pure Chemical Industries (Osaka, Japan).

V. RESULTS

Table 17 shows changes in the fatty acid composition at the C-2 position of GalDG before and after the incubation for 7.5 h in the presence of cerulenin. As elucidated in a previous paper [8], only 16:0 and 16:1 were esterified to the C-2 position under these conditions. The total amount of the C_{16} acids decreased from 14 to 13 μmol/liter during the incubation at both 38 and 22°C. This indicates that cerulenin, an inhibitor of the fatty acid synthesis [162], suppressed the synthesis of GalDG (Chapter 2). During the incubation, the relative content of 16:0 decreased from 47 to 32 % at 38°C and to 21 % at 22°C, and that of 16:1 increased from 53 to 68 % at 38°C and to 79 % at 22°C. These findings suggest that C_{18}/16:0 species were converted to C_{18}/16:1 during the incubation at 38 and 22°C. The ratio, B/C, in Eq.(2) (p.92) was determined from the values in Table 17. It was 3.53 and 2.04 in the incubation at 38 and 22°C, respectively.

Figure 15 shows mass spectra of unenriched 1-TMS-2-(16:0)-3-tetrakis(TMS)galactosylglycerol and 1-TMS-2-(16:1)-3-tetrakis(TMS)galactosylglycerol. Their molecular ions (m/e 852 and 850) were not detected.

Table 17. Changes in the fatty acid composition at the C-2 position of GalDG before and after incubation in the presence of cerulenin (10 μg/ml).

Temperature* (°C)	Time* (h)	Percentage 16:0	Percentage 16:1	Total amount (μmol/liter)
Before incubation		47	53	14
38	7.5	32	68	13
22	7.5	21	79	13

*Temperature and time after addition of cerulenin.

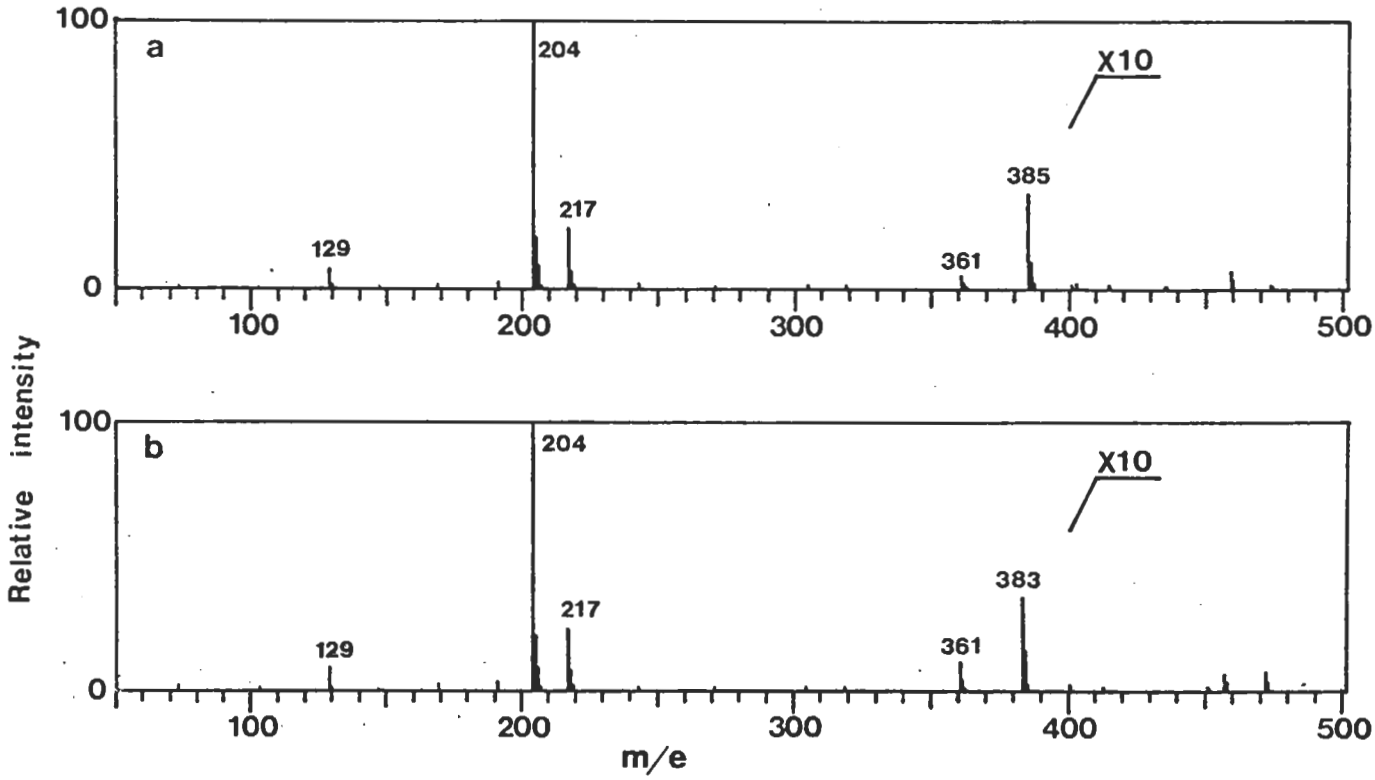

Fig. 15. Mass spectra of 1-TMS-2-acyl-3-tetrakis(TMS)galactosylglycerols without ^{13}C-enrichment.

a, 1-TMS-2-(16:0)-3-tetrakis(TMS)galactosylglycerol;

b, 1-TMS-2-(16:1)-3-tetrakis(TMS)galactosylglycerol.

The fragment ions with m/e 204, 217 and 361 were derived from the trimethylsilylated galactose moiety. The fragment ion derived from 1-TMS-2-(16:0)-3-tetrakis(TMS)galactosylglycerol having m/e 385 and that derived from the 2-(16:1) counterpart having m/e 383 contained the TMS group at the C-1 position, the acyl group at the C-2 position and the glycerol backbone. We name them TMS-Gly-16:0 and TMS-Gly-16:1 (or TMS-Gly-C_{16}) fragments.

Incorporation of ^{13}C into Gly-C_{16}

Figures 16a and b show mass distributions of the unenriched TMS-Gly-16:0 and TMS-Gly-16:1 fragments. The main peaks at m/e 385 in TMS-Gly-16:0 and at m/e 383 in TMS-Gly-16:1 represent fragments composed of ^{12}C, ^{1}H, ^{16}O and ^{28}Si. These m/e values are defined as M_0. Peaks having m/e values larger than M_0 are produced by the same fragments containing the isotopes of ^{13}C, ^{2}H, ^{17}O, ^{18}O, ^{29}Si and ^{30}Si of natural abundance. Peaks having m/e 401, 402, 403 and 404 in TMS-Gly-16:0 and 398, 399, 400, 401 and 402 in TMS-Gly-16:1 were assigned to fragments of a different structure. Figure 16c shows mass distribution of TMS-Gly-16:0 derived from the cells fed with $Na_2{}^{13}CO_3$ at 38°C for 2.5 h. Peaks having m/e from 390 to 400 were distinct. The intensity was maximal at m/e 396. TMS-Gly-16:1 derived from the same cells contained insignificant amounts of such high m/e peaks (Fig. 16d). After the incubation at 38°C for 7.5 h, the mass spectrum of TMS-Gly-16:0 (Fig. 16e) remained almost unchanged, while the peaks of TMS-Gly-16:1 having m/e from 388 to 398 were apparent (Fig. 16f). This is accounted for by the desaturation of 16:0 of the enriched GalDG during the incubation time. After the incubation at 22°C for 7.5 h, the mass spectrum of TMS-Gly-16:0 (Fig.16g) remained almost unchanged, whereas TMS-Gly-16:1 (Fig. 16h) came to

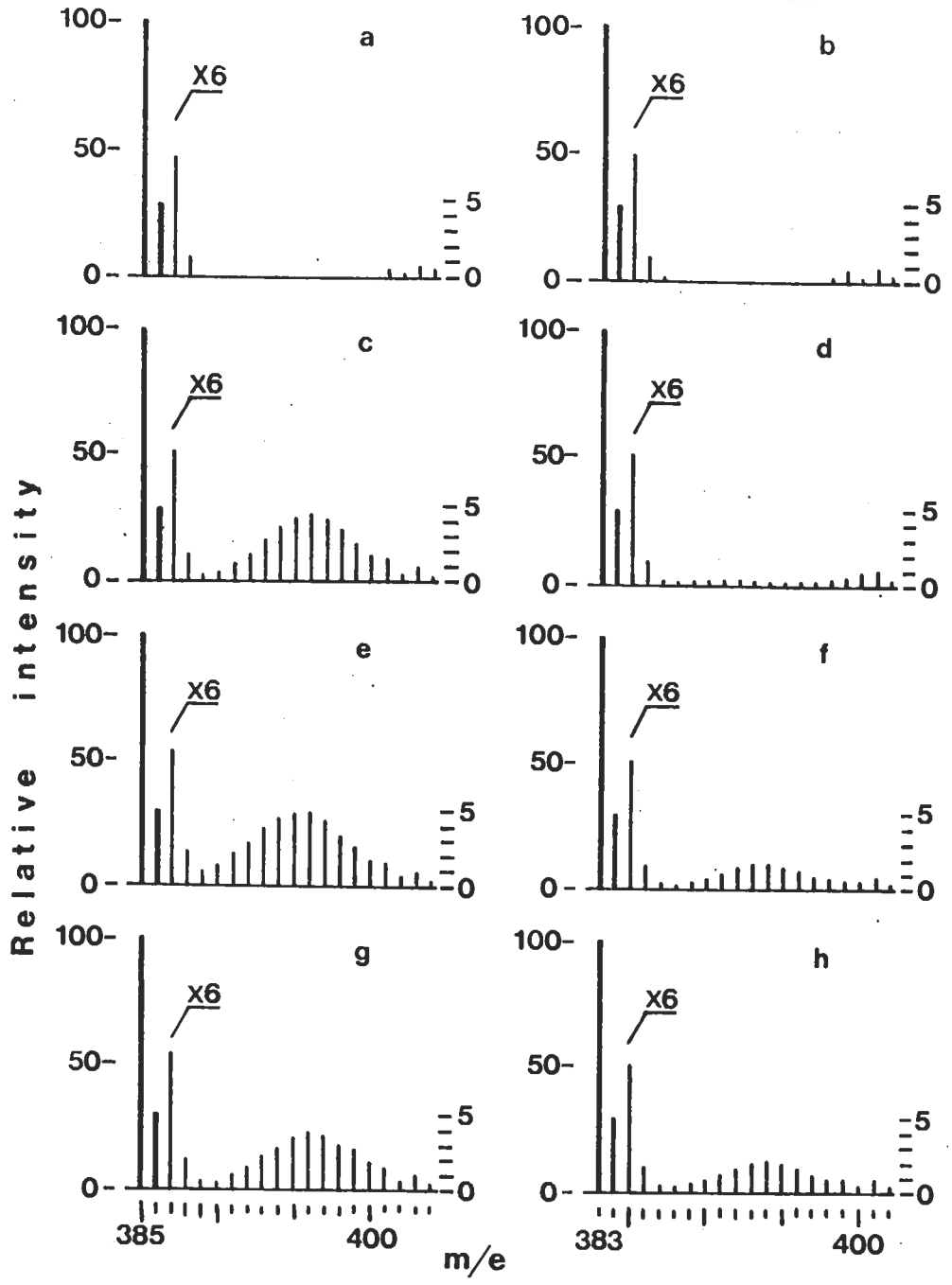

Fig. 16. Mass spectra of 1-TMS-2-acyl-3-tetrakis(TMS)galactosylglycerols derived from GalDG in the cells before and after the ^{13}C-feeding. TMS-Gly-C_{16} fragment ions are shown.
a, 16:0-species before the ^{13}C-feeding; b, 16:1-species before the ^{13}C-feeding; c 16:0-species after the ^{13}C-feeding for 2.5 h; d, 16:1-species after the ^{13}C-feeding for 2.5 h; e, 16:0-species after the incubation for 7.5 h at 38°C; f, 16:1-species after the incubation for 7.5 h at 38°C; g, 16:0-species after the incubation for 7.5 h at 22°C; h, 16:1-species after the incubation for 7.5 h at 22°C.

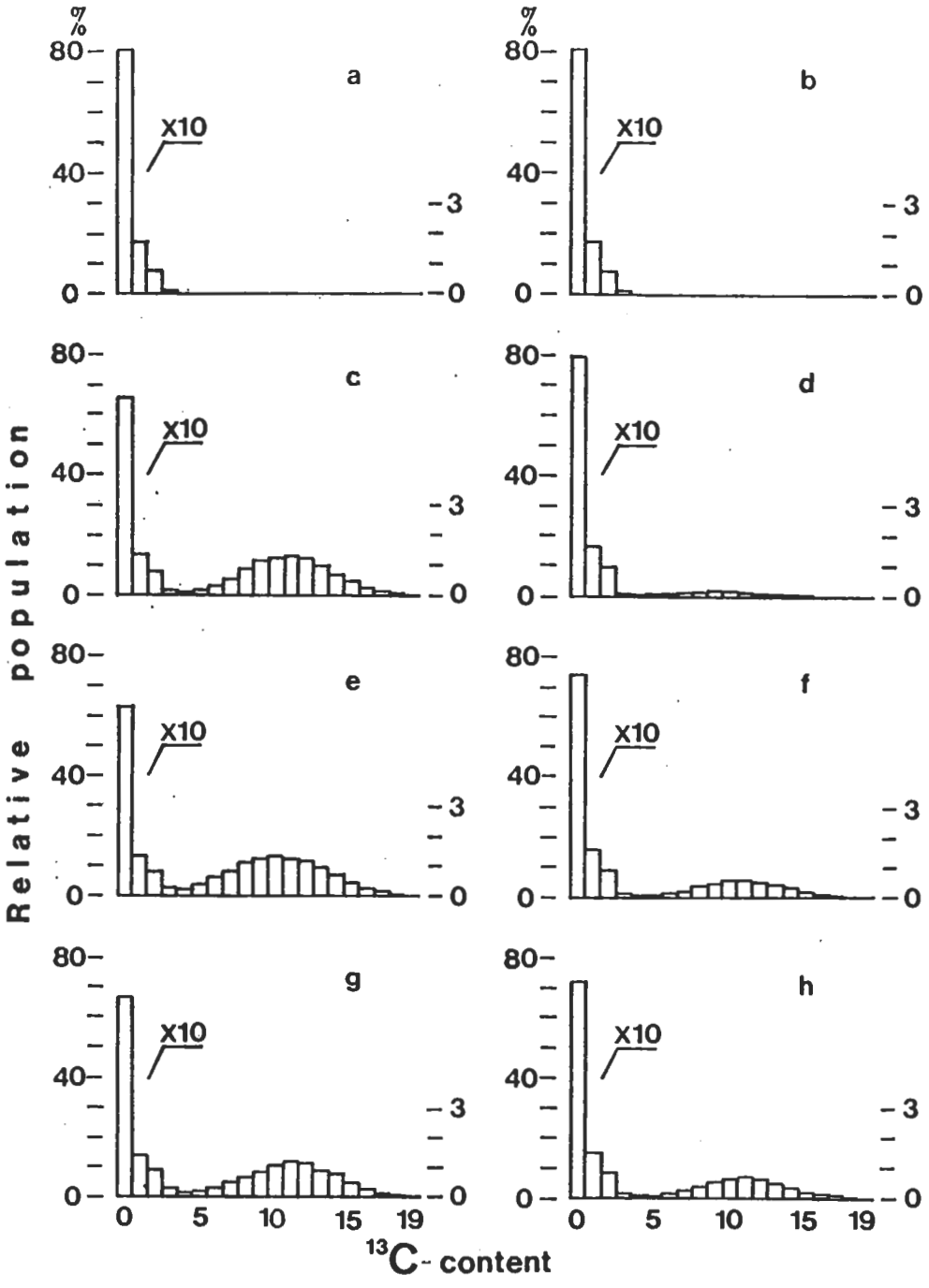

Fig. 17. Histograms of ^{13}C-content distributions in Gly-C_{16} moiety of GalDG. The distributions in unenriched Gly-C_{16} (a and b) were calculated from the natural abundance of ^{13}C. The distributions in Gly-C_{16} after the ^{13}C-feeding (c-h) were calculated from the mass spectra in Fig. 16 according to procedure in Appendix 1. Figure notations are the same as in Fig. 16.

contain greater amounts of peaks having m/e from 388 to 398 than after the incubation at 38°C. This is accounted for by an accelerated desaturation of 16:0 in GalDG during the incubation at 22°C.

The shift of mass peaks to the greater m/e values arises not only from ^{13}C in the Gly-C_{16} part but also from the natural abundance of ^{13}C, ^{2}H, ^{29}Si and ^{30}Si in the TMS group and ^{2}H, ^{17}O and ^{18}O in the Gly-C_{16} part. By eliminating the influences of these isotopes according to the mathematical treatment described in Appendix 1, the ^{13}C-content distributions in Gly-C_{16} (Figs. 17c-h) were calculated from the observed mass spectra (Figs. 16c-h). The ^{13}C-content distributions of the unenriched Gly-C_{16} (Figs. 17a and b) were calculated from the natural abundance of ^{13}C.

The changes in the ^{13}C-content distributions in Fig. 17 indicate that the relative content of Gly-16:0 having four to nineteen ^{13}C atoms amounted to 19 % of the total after the ^{13}C-feeding for 2.5 h (Fig. 17c). At this stage, only 2 % of Gly-16:1 were enriched with ^{13}C (Fig. 17d).

During the incubation for 7.5 h at 38 and 22°C, the ^{13}C-content distribution in Gly-16:0 changed only slightly (Fig. 17e). The relative contents of Gly-16:1 having four to nineteen ^{13}C atoms amounted to 8.0 and 9.5 % after the incubation for 7.5 h at 38 and 22°C, respectively. These contents of ^{13}C-enriched molecules were consistent with the values expected from the decrease in 16:0 and the increase in 16:1 in Table 17.

Incorporation of ^{13}C into C_{16} acids

As described in THEORETICAL, the ^{13}C-content distributions in Gly and C_{16} are necessary in estimating the ^{13}C-content distributions in the U-U, E-U, U-E and E-E combinations of Gly-C_{16}. Figure 18 shows mass spectra of the molecular ion of methyl palmitate (M^+) and the fragment ions of methyl palmitoleate ($(M-31)^+$ and $(M-32)^+$) in GalDG before and after the

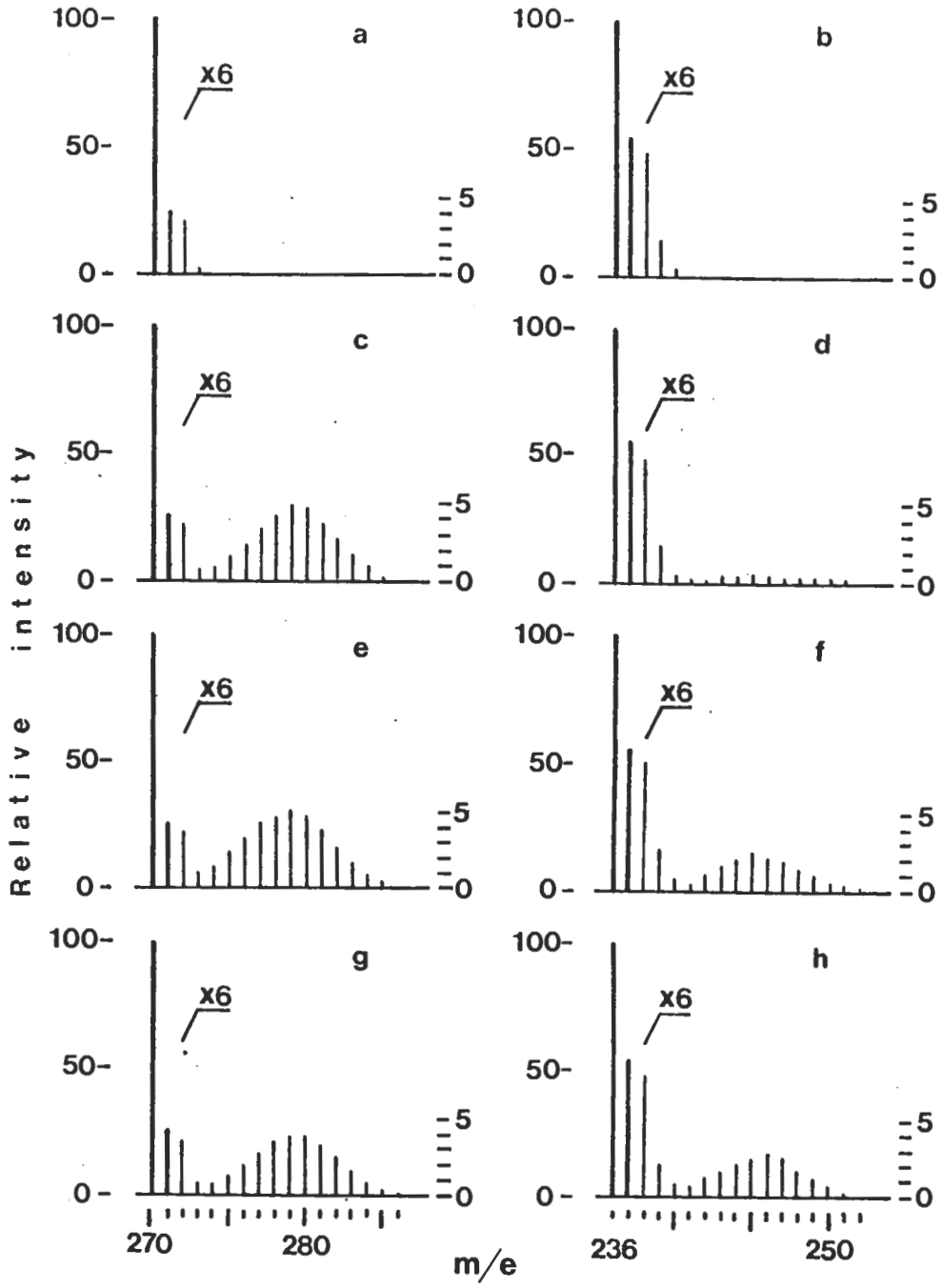

Fig. 18 Mass spectra of fatty acid methyl esters derived from GalDG in the cells before and after the ^{13}C-feeding. Molecular ions of methyl palmitate, and $(M-31)^+$ and $(M-32)^+$ fragment ions of methyl palmitoleate are shown. a, 16:0 before the feeding; b, 16:1 before the feeding; c, 16:0 after the ^{13}C-feeding for 2.5 h; d, 16:1 after the ^{13}C-feeding for 2.5 h; e, 16:0 after the incubation for 7.5 h at 38°C; f, 16:1 after the incubation for 7.5 h at 38°C; g, 16:0 after the incubation for 7.5 h at 22°C; h, 16:1 after the incubation for 7.5 h at 22°C.

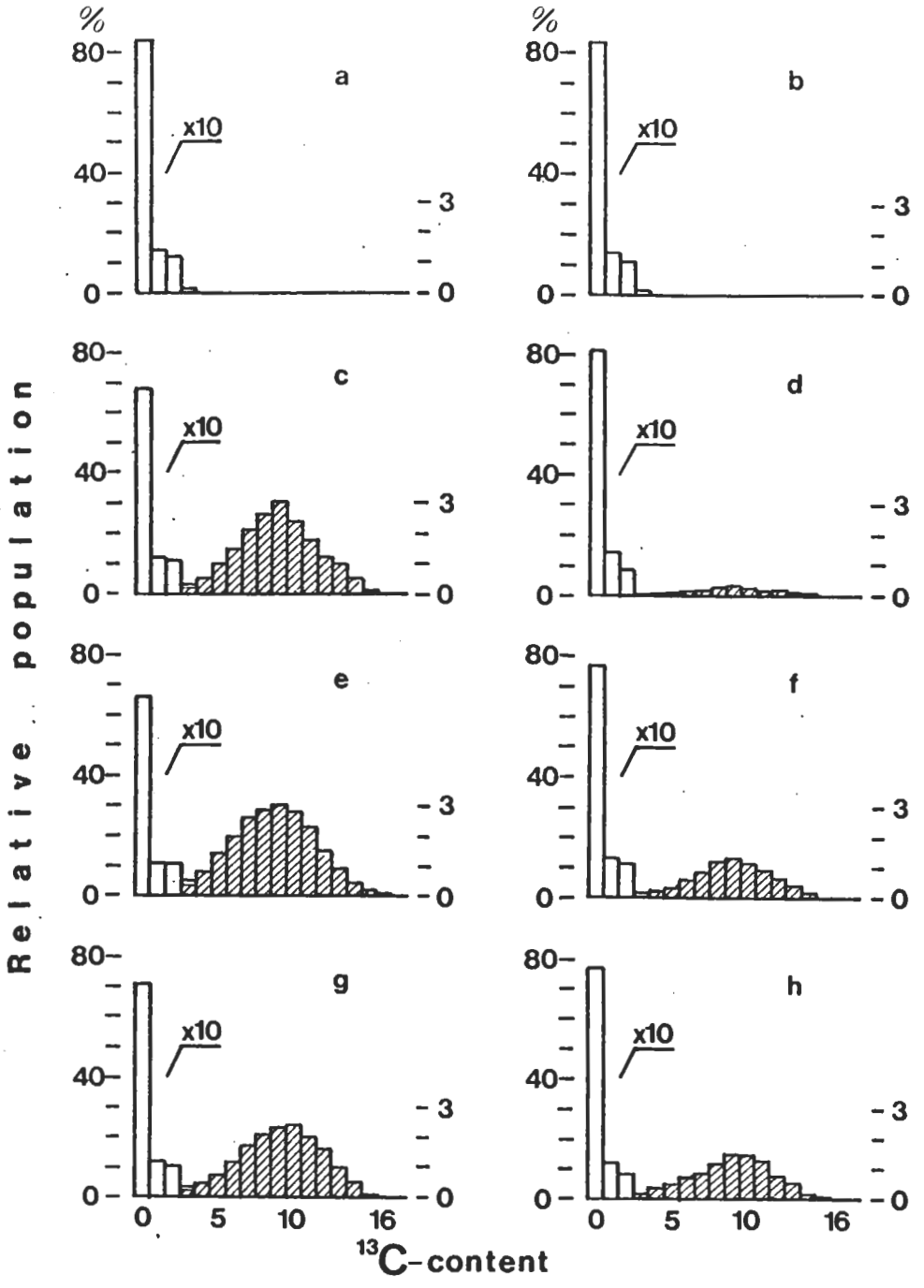

Fig. 19 Histograms of ^{13}C-content distributions in fatty acids of GalDG. The distributions in unenriched C_{16} were calculated from the natural abundance of ^{13}C. The distributions in C_{16} after the ^{13}C-feeding were calculated from the mass spectra in Fig. 18 according to the procedure in Appendix 1. The shaded parts of the bars represent the ^{13}C-content distribution of enriched fatty acids. Figure notations are the same as in Fig. 18.

^{13}C-feeding. In the mass spectrum of methyl palmitoleate, the mass peaks of the fragment ions were far more intense than those of the molecular ion, and therefore were used for the determination of the ^{13}C-content distribution in 16:1.

The main peak at m/e 270 of unenriched methyl palmitate (Fig. 18a) was due to the molecular ion having ^{12}C, ^{1}H and ^{16}O but no heavier isotopes, and the peaks at m/e 271, 272 and 273 were due to the natural abundance of ^{13}C, ^{2}H, ^{17}O and ^{18}O. The peaks at m/e 236 of unenriched methyl palmitoleate was a fragment of $C_{15}H_{28}CO$ having ^{12}C, ^{1}H and ^{16}O. The peak at m/e 237 originated from a fragment of $C_{15}H_{29}CO$ having ^{12}C, ^{1}H and ^{16}O and the fragment of $C_{15}H_{28}CO$ in which one of the atoms was replaced by ^{13}C, ^{2}H , ^{17}O or ^{18}O. Isotope peaks of these two fragments were mixed to constitute the peaks at m/e 238 to 240. The mass spectra of fatty acid methyl esters (Fig. 18) were converted to the ^{13}C-content distributions (Fig. 19) according to the mathematical treatment as described in Appendix 1.

The changes in the ^{13}C-content distributions in 16:0 and 16:1 after the ^{13}C-feeding and the incubation were illustrated in Fig. 19. On the assumption that the ^{13}C-content distributions of the unenriched 16:0 and 16:1 are unaltered during the ^{13}C-feeding and the subsequent incubation, and that the population at zero ^{13}C-content is composed only of the unenriched C_{16}, it is possible to estimate the ^{13}C-content distributions of enriched (E) and unenriched (U) groups of C_{16}. The results thus obtained are presented in Fig. 19 by the shaded and open bars representing enriched and unenriched C_{16}, respectively.

After the ^{13}C-feeding for 2.5 h (Fig. 19c), 16:0(E) amounted to 19% and the content of ^{13}C in 16:0(E) was 9.0 or 56 %. At this stage, 16:1(E)

amounted to only 2 % of 16:1 (Fig. 19d). After the incubation for 7.5 h at 38°C, the relative amount of 16:0(E) remained almost the same as before the incubation (Fig. 19e), whereas 16:1(E) amounted to 8.0 % of 16:1 (Fig. 19f). After the incubation for 7.5 h at 22°C, the ^{13}C-content distribution in 16:0 (Fig. 19g) changed only slightly, whereas 16:1(E) amounted to 9.5 % of 16:1 (Fig. 19h). The ^{13}C-content distributions in 16:1(E) and 16:0(E) showed almost identical patterns with a maximum at ^{13}C-content 9.

Incorporation of ^{13}C into glycerol

Figure 20 shows mass spectra of tris(TMS)glycerol derived from GalDG before and after the ^{13}C-feeding. In the mass spectrum of unenriched tris(TMS)glycerol, peaks with m/e 217 and 218 were due to $C_3H_4(O\text{-}TMS)_2$ and $C_3H_5(O\text{-}TMS)_2$, respectively, having ^{12}C, ^{1}H, ^{16}O and ^{28}Si. Because of a large content of heavier isotopes, especially ^{29}Si and ^{30}Si, four other peaks appeared at m/e 219, 220, 221 and 222. The mass spectra of tris(TMS)glycerol before and after the ^{13}C-feeding were converted to the ^{13}C-content distributions (Fig. 21) according to the mathematical treatment described in Appendix 1.

Figure 21 shows the ^{13}C-content distributions of glycerol derived from GalDG before and after the ^{13}C-feeding. The ^{13}C-content distributions of Gly(U) and Gly(E) overlapped each other. They were, therefore, separated on the assumption that the ratios of Gly(E) to Gly(U) were the same as those of $C_{16}(E)$ to $C_{16}(U)$ in Fig. 19. The ^{13}C-content distributions of Gly(E) and Gly(U) are shown by the shaded and open bars in Fig.21. The ^{13}C-content distributions of Gly(E) were similar to one another in that the ^{13}C-content ranged from 0 to 3 with a maximum at ^{13}C-content 2.

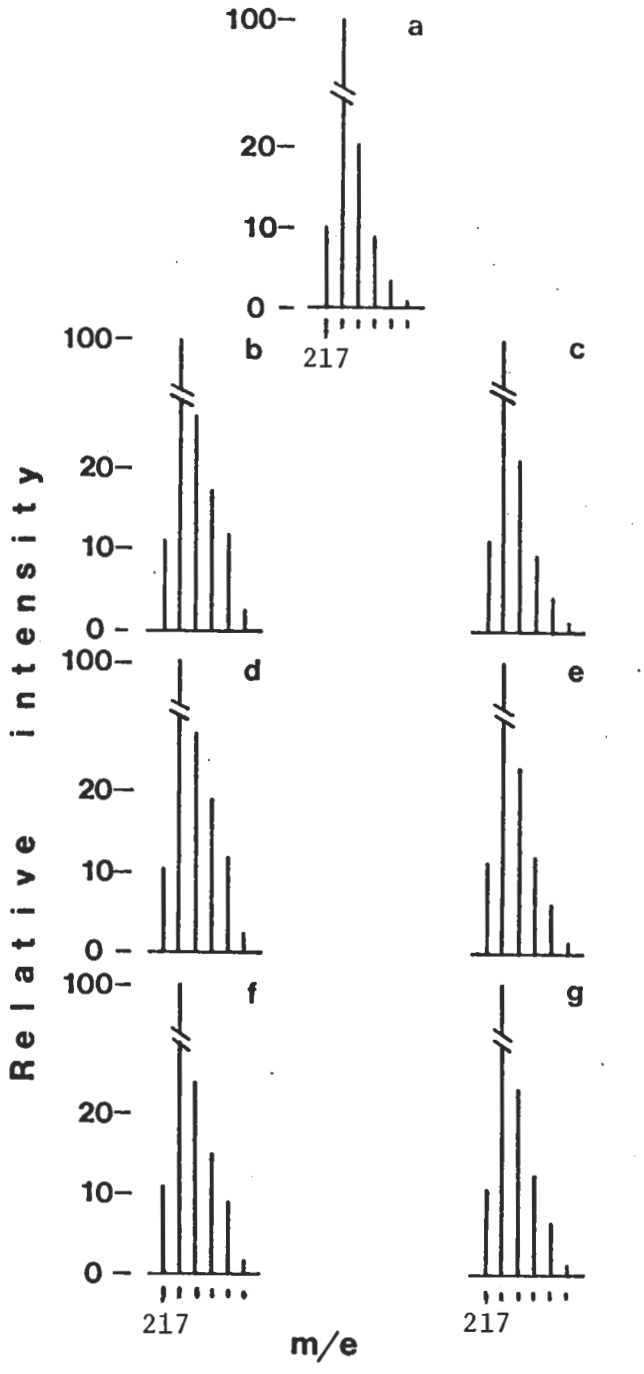

Fig. 20. Mass spectra of tris(TMS)glycerol derived from GalDG in the cells before and after the ^{13}C-feeding. Fragment ions of bis(TMS)glycerol are shown. a, Glycerol before the ^{13}C-feeding; b, Glycerol from the 2-(16:0)-species after the ^{13}C-feeding for 2.5 h; c, Glycerol from the 2-(16:1)-species after the ^{13}C-feeding for 2.5 h; d, Glycerol from the 2-(16:0)-species after the incubation for 7.5 h at 38°C; e, Glycerol from the 2-(16:1)-species after the incubation for 7.5 h at 38°C; f, Glycerol from the 2-(16:0)-species after the incubation for 7.5 h at 22°C; g, Glycerol from the 2-(16:1)-species after the incubation for 7.5 h at 22°C.

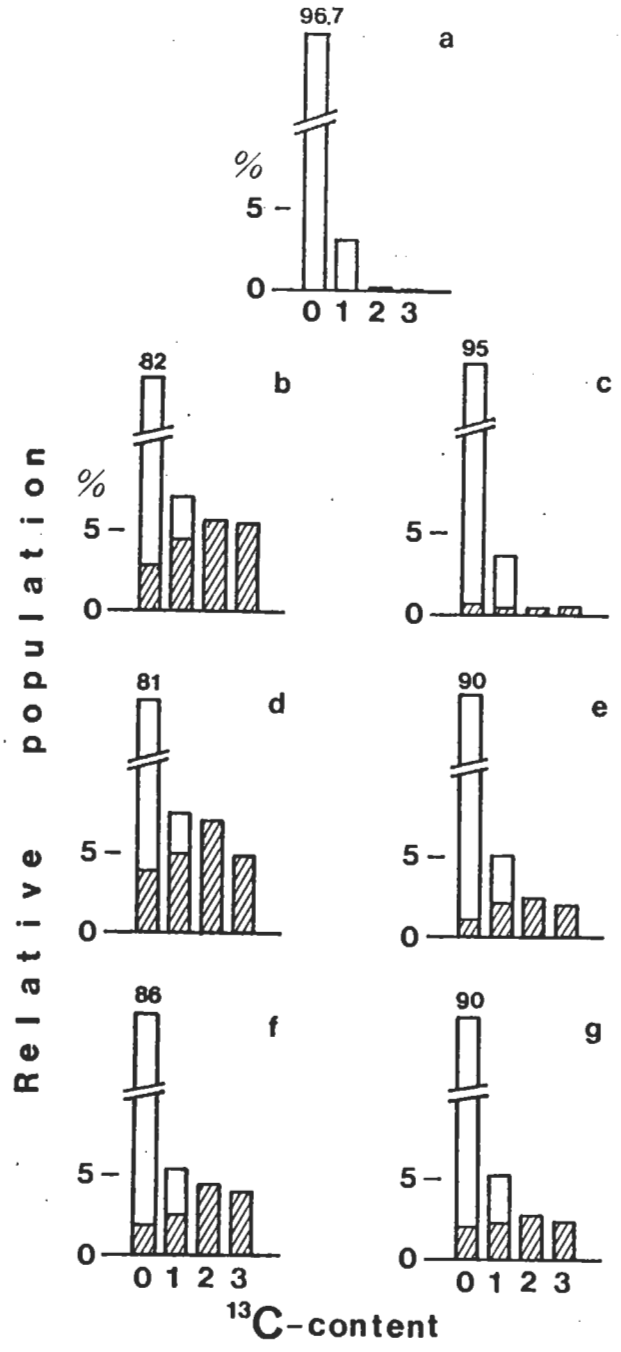

Fig. 21 Histograms of ^{13}C-content distributions in glycerol of GalDG. The distribution in unenriched glycerol was calculated from the natural abundance of ^{13}C. The distributions in glycerol after the ^{13}C-feeding were calculated from the mass spectra in Fig. 20 according to the procedure described in Appendix 1. The shaded parts of the bars represent the ^{13}C-content distribution of enriched glycerol. Figure notations are the same as in Fig. 20.

Isotope combination analysis

From the ^{13}C-content distributions in C_{16}(U), C_{16}(E), Gly(U) and Gly(E), the ^{13}C-content distributions in the U-U, E-U, U-E and E-E combinations of Gly-C_{16} after the ^{13}C-feeding and the subsequent incubation were calculated according to the mathematical treatment described in Appendix 2. An example for the ^{13}C-content distributions in the isotope combinations of Gly-16:0 derived from the cells fed with ^{13}C for 2.5 h is shown in Fig. 22. In Gly-16:1 after the ^{13}C-feeding for 2.5 h, in which the relative amounts of 16:1(E) (Fig. 19d) and Gly(E) (Fig. 21c) were very small, their ^{13}C-content distributions were not precisely determined. In this case, the distributions in 16:0(E) and Gly(E) in Gly-16:0 after the ^{13}C-feeding for 2.5 h were used in calculating the ^{13}C-content distributions in the E-U, U-E and E-E combinations of Gly-16:1.

As described in THEORETICAL, the observed ^{13}C-content distribution in Gly-C_{16} (Fig. 17) should be a linear combination of the ^{13}C-content distributions calculated for the U-U, E-U, U-E and E-E combinations of Gly-C_{16}. For the determination of contributions of the four isotope combinations, the observed distribution was simulated by the least square method with the distributions of isotope combinations. The result presented in Fig. 23 indicates that the contributions of U-E and E-U combinations in composing Gly-C_{16} were very small in all the cases.

Mechanism for the desaturation of 16:0

The contributions of the four isotope combinations in simulating the observed ^{13}C-content distributions of Gly-C_{16} are shown in Table 18. Gly-16:0 from the cells fed with ^{13}C for 2.5 h was composed of 80.6 % U-U, 1.1 % E-U, 0.9 % U-E and 17.4 % E-E, whereas Gly-16:1 was composed mainly of U-U. Therefore, the necessary condition considered in THEORETICAL

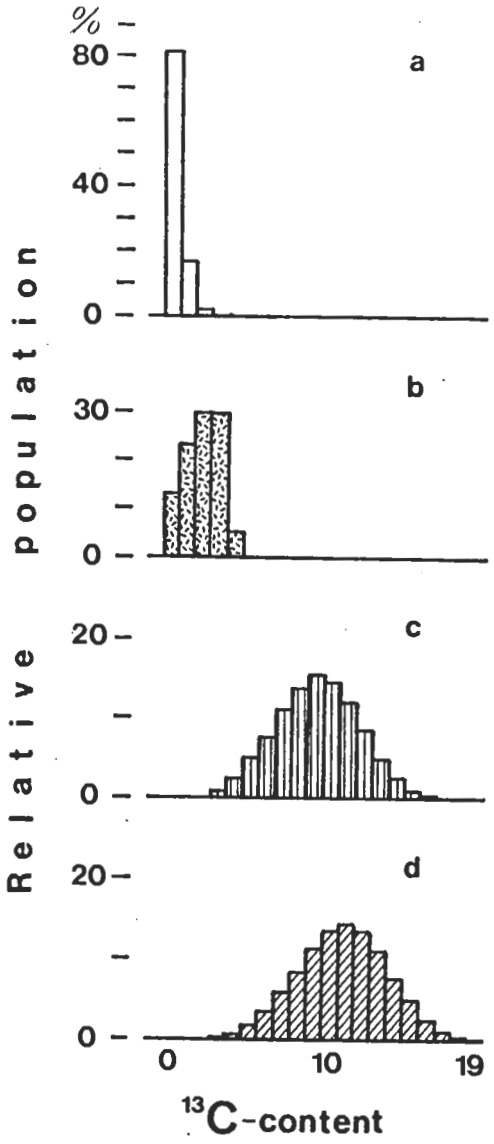

Fig. 22. The ^{13}C-content distributions in the four isotope combinations of Gly-16:0 of GalDG derived from the cells fed with ^{13}C for 2.5 h at 38°C. The distributions were calculated from the ^{13}C-content distributions in 16:0(U) in Fig. 19a, 16:0(E) in Fig. 19c (shaded bars), Gly(U) in Fig. 21a and Gly(E) in Fig. 21b (shaded bars) according to the mathematical treatment described in Appendix 2. a, U-U; b, E-U; c U-E; d, E-E.

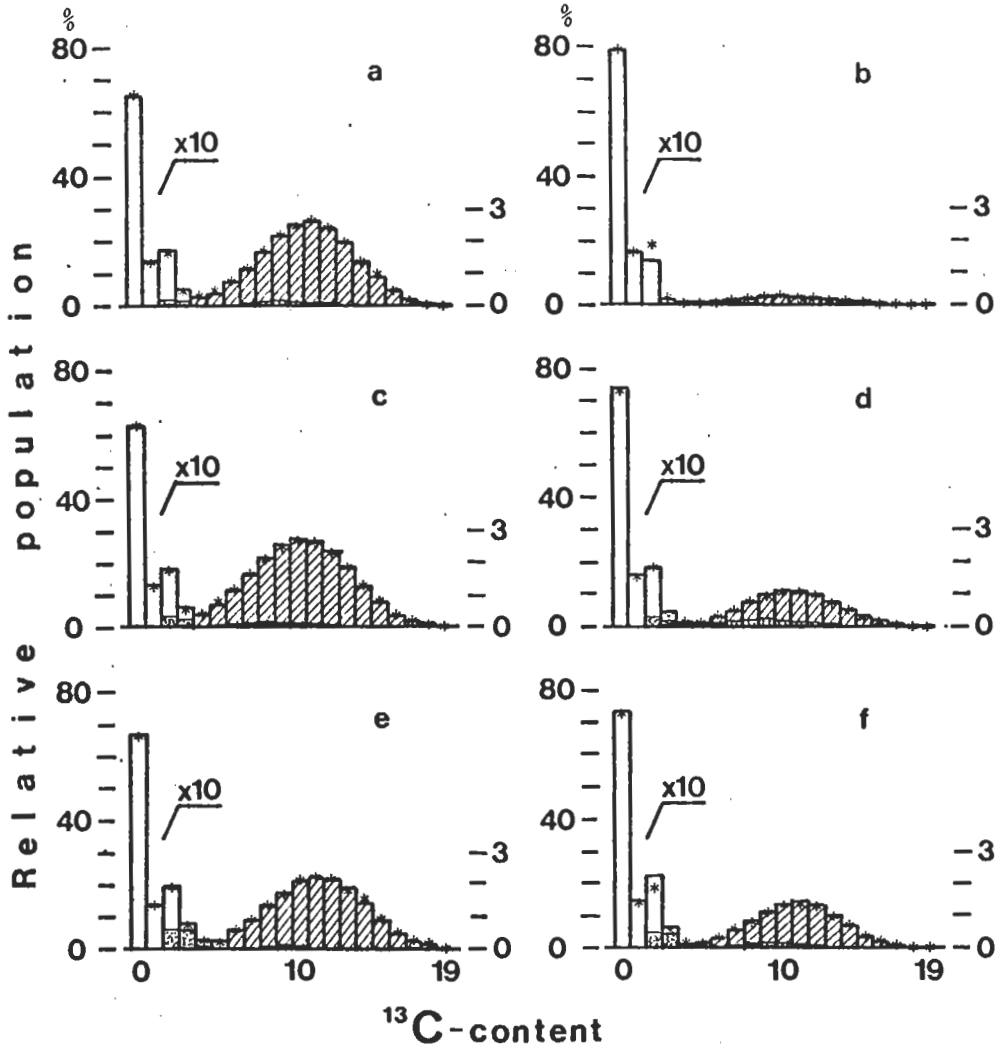

Fig. 23. Graphic representation of the result of the isotope combination analysis. The ^{13}C-content distribution of Gly-C_{16} moiety of GalDG was simulated by the least square method with the ^{13}C-content distributions of the U-U, E-U, U-E and E-E combinations. The experimentally observed distributions (Fig. 17) are expressed by the asterisks. Contributions of the four isotope combinations are illustrated by the same marks as in Fig. 22. a, Gly-16:0 after the ^{13}C-feeding for 2.5 h; b, Gly-16:1 after the ^{13}C-feeding for 2.5 h; c, Gly-16:0 after the incubation for 7.5 h at 38°C; d, Gly-16:1 after the incubation for 7.5 h at 38°C; e, Gly-16:0 after the incubation for 7.5 h at 22°C; f, Gly-16:1 after the incubation for 7.5 h at 22°C.

Table 18. The result of isotope combination analysis of Gly-C_{16} moiety of GalDG. The cells were fed with $Na_2{}^{13}CO_3$ for 2.5 h at 38°C, and then incubated for 7.5 h at 38 and 22°C in the presence of cerulenin. Relative contributions of isotope combinations in simulating the observed ^{13}C-content distributions of Gly-C_{16} are presented in percent.
The values for h (observed) were (E-U + U-E)/(U-U + E-U + U-E + E-E). The theoretically predicted values for h (direct desaturation) and h(deacylation-desaturation-reacylation) were calculated according to Eq. (1) from the relative contributions of the four isotope combinations before the incubation.

	Before incubation		After incubation at 38°C		After incubation at 22°C	
	Gly-16:0	Gly-16:1	Gly-16:0	Gly-16:1	Gly-16:0	Gly-16:1
U-U	80.6	97.5	77.8	91.4	81.8	88.9
E-U	1.1	0.8	1.4	1.4	0.5	0.8
U-E	0.9	0.5	1.3	0.9	1.7	1.8
E-E	17.4	1.8	19.5	6.3	16.0	8.5
h(observed) x 100	2.0	1.3	2.7	2.3	2.2	2.6
Theoretical calculation						
h(direct desaturation) x 100				1.5		1.5
h(deacylation-desaturation-reacylation) x 100				7.7		10.7

(the left side in Fig. 13D) was rather well established after the ^{13}C-feeding for 2.5 h. After the incubation for 7.5 h, during which time a part of 16:0 was converted to 16:1, Gly-16:1 came to contain 1.4 % E-U, 0.9 % U-E and 6.3 % E-E at 38°C, and 0.8 % E-U, 1.8% U-E and 8.5 % E-E at 22°C. By comparing Tables 16 and 18, the following values were determined: x=0.011, y=0.009, z=0.174, u=0.008, v=0.005, w=0.018. The ratio, B/C, was 3.53 for the incubation at 38°C and 2.04 for the incubation at 22°C (Table 17). In substituting these values for the variables in Eq.(1), the predicted value for h at 38°C is 1.5 in the direct desaturation and 7.7 in the deacylation-desaturation-reacylation, that at 22°C is 1.5 in the direct desaturation and 10.7 in the deacylation-desaturation-reacylation. The observed values for h were close to the predicted values in the direct desaturation. We conclude, therefore, that 16:0 in GalDG was desaturated to 16:1 mainly by the direct desaturation both in the isothermal growth at 38°C and after the temperature shift to 22°C.

The relative contributions of the direct desaturation and the deacylation-desaturation-reacylation are quantitatively estimated by substituting the observed values in Eq. (2). d is calculated to be 0.14 at 38°C and 0.11 at 22°C. It is concluded, therefore, that the direct desaturation accounts for almost 90 % of the desaturation of 16:0 esterified to the C-2 position of GalDG at both 38 and 22°C.

VI. DISCUSSION

The direct desaturation of fatty acid in GalDG is definitely demonstrated for the first time. Several *in vivo* studies using ^{14}C have suggested that GalDG might be the substrate for the desaturation of fatty acids in higher plants [60, 61], *Chlorella* [25] and *Anabaena* [15].

These time course studies could only give indirect evidences and could not exclude a possibility of the deacylation-desaturation-reacylation mechanism.

The isotope combination analysis allowed us to study the direct desaturation *in vivo*. The use of concentrated ^{13}C in labeling both of the glycerol and the acyl group cannot be replaced by the double labeling with ^{14}C and ^{3}H, since a possible exchange of acyl groups between the lipid molecules cannot be detected. For example, a mixture of ^{14}C-palmitoyl-^{3}H-glycerol and unlabeled palmitoyl-glycerol is indistinguishable from a mixture of ^{14}C-palmitoyl-^{1}H-glycerol and ^{12}C-palmitoyl-^{3}H-glycerol by means of radioactivity. The isotope combination analysis is based on the same principle as the "isotope cross over method" utilized in organic chemistry to discriminate intra- and intermolecular reactions [168]. In applying the method to the biological systems, a necessary condition for the initial state, *i.e.*, coexistence of U-U and E-E but no U-E and E-U combinations, has to be established. The Gly-16:0 moiety of GalDG in *A. variabilis* which is fed with $Na_2{}^{13}CO_3$ for 2.5 h at 38°C satisfies this condition.

The role of desaturation of 16:0 has been demonstrated in the temperature acclimation in *A. variabilis* [28] and now the desaturation is proved to be the direct one. The direct desaturation of pre-existing acyl residue might be the most effective way in increasing the fatty acid unsaturation. It is therefore quite natural that the direct desaturation participates in the rapid changes in fatty acid composition [28] after the downward temperature shift.

VII. APPENDIX 1 Conversion of the mass spectra to the ^{13}C-content distributions

Because of the natural abundance of stable isotopes, ^{13}C, ^{2}H, ^{17}O, ^{18}O, ^{29}Si and ^{30}Si, mass peaks of molecular and fragment ions having m/e values greater than the base peak appear in the mass spectrum. Therefore, the observed mass spectra do not directly correspond to the ^{13}C-content distributions of Gly-C_{16}, C_{16} and Gly. According to a mathematical treatment in the followings, the mass spectra are converted to the ^{13}C-content distributions.

TMS-Gly-16:0 fragment

The ^{13}C-content distribution in Gly-16:0 was calculated from the mass spectrum of TMS-Gly-16:0 fragment. The TMS-Gly-16:0 fragment contains 19 carbon atoms that can be enriched with ^{13}C. The mass of its base peak, M_0, is 385.

As shown in Figs. 16a and b, the mass peaks having m/e from 401 to 404 originate from fragments other than TMS-Gly-16:0. Therefore, at first, the peaks in Figs. 16 c-h were corrected for the interference of these fragments.

The observed mass spectrum is normalized so that the intensity, I_i, of mass peak having m/e (M_0+i) satisfies:

$$\sum_{i=0}^{19} I_i = 1, \qquad \text{----------(3)}$$

The ^{13}C-content distribution is represented by the proportions of molecules containing j ^{13}C atoms, D_j, which satisfy:

$$\sum_{j=0}^{19} D_j = 1. \qquad \text{----------(4)}$$

I_i and D_j are related by splitting coefficients, c_k^j , the values for which depend on the number of ^{2}H, ^{17}O, ^{18}O, ^{29}Si and ^{30}Si involved in the whole molecule and of ^{13}C in the TMS group. c_k^j is defined as a proportion of the fragment having j ^{13}C atoms and the m/e value, (M_0+j+k). It satisfies:

$$\sum_{k=0}^{19} c_k^j = 1 . \qquad \text{----------(5)}$$

It is assumed that c_k^j is equal to c_k^0. This is reasonable since the contents of the above mentioned isotopes are independent of j, the number of ^{13}C atoms involved in the Gly-C_{16} part. c_k^0 is re-written as c_k. Then, I_i, D_j and c_k should satisfy the following set of simultaneous equations:

$$I_0 = c_0 D_0 \qquad \text{----------(6-0)}$$

$$I_1 = c_1 D_0 + c_0 D_1 \qquad \text{----------(6-1)}$$

$$I_2 = c_2 D_0 + c_1 D_1 + c_0 D_2 \qquad \text{----------(6-2)}$$

.....

$$I_i = c_i D_0 + c_{i-1} D_1 + \ldots\ldots\ldots\ldots + c_0 D_i \qquad \text{----------(6-i)}$$

.....

$$I_{19} = c_{19} D_0 + c_{18} D_1 + \ldots\ldots\ldots\ldots\ldots + c_1 D_{18} + c_0 D_{19} . \qquad \text{----(6-19)}$$

c_k is determined from I_i in the unenriched TMS-Gly-16:0 and D_j for the unenriched Gly-16:0. The observed values for I_i were:

$I_0 = 0.7226$, $I_1 = 0.2088$, $I_2 = 0.0578$, $I_3 = 0.0097$, $I_4 = 0.0011$ and $I_i < 10^{-3}$ for $i \geq 5$.

The natural abundance of ^{13}C is 1.107 %. D_j is calculated according to a binomial distribution:

$$D_j = \binom{19}{j} \cdot (0.01107)^j \cdot (0.98893)^{19-j} \qquad \text{----------(7)}$$

Then,

$D_0 = 0.8094$, $D_1 = 0.1721$, $D_2 = 0.0173$, $D_3 = 0.0011$, and $D_j < 10^{-4}$ for $j \geq 4$.

With these values for I_i and D_j for the unenriched fragments, c_k is determined by solving Eqs. (6-0) to (6-4).

$c_0 = 0.8928$, $c_1 = 0.0681$, $c_2 = 0.0378$, $c_3 = 0.0013$ and $c_k < 10^{-3}$ for $k \geq 4$.

From c_k thus determined and I_i observed for the TMS-Gly-16:0 fragment of GalDG derived from the cells fed with ^{13}C, D_j (the ^{13}C-content distribution in Gly-16:0) is calculated by solving the set of Eqs. (6-0) to (6-19). The result is presented in Figs. 17c, e and g.

TMS-Gly-16:1 fragment

The ^{13}C-content distribution in Gly-16:1 was calculated from the mass spectrum of TMS-Gly-16:1 fragment in a way similar to that in Gly-16:0. The result is presented in Figs. 17d, f and h.

Molecular ion of methyl palmitate

The ^{13}C-content distribution in 16:0 was calculated from the mass spectrum of methyl palmitate. The molecular ion of this molecule contains 16 carbon atoms that can be enriched with ^{13}C and M_0 is 270. The observed mass spectrum is represented by the normalized peak intensities, I_i. D_j and c_k are defined as in the TMS-Gly-16:0 fragment. I_i, D_j and c_k should satisfy a set of Eqs. (6-0) to (6-16).

c_k is determined from I_i for unenriched methyl palmitate and D_j for unenriched 16:0. The observed values for I_i (Fig. 18a) were:

$I_0 = 0.7732$, $I_1 = 0.1979$, $I_2 = 0.0263$, $I_3 = 0.0025$ and
$I_i < 10^{-3}$ for $i \geq 4$.

D_j is calculated according to the following equation:

$$D_j = \binom{16}{j} \cdot (0.01107)^j \cdot (0.98893)^{16-j} \qquad \text{----------(8)}$$

Then,

$D_0 = 0.8369$, $D_1 = 0.1499$, $D_2 = 0.0126$, $D_3 = 0.0007$ and
$D_j < 10^{-4}$ for $j \geq 4$.

With these values, c_k is determined for the case of unenriched 16:0 by solving Eqs. (6-0) to (6-3).

$c_0 = 0.9239$, $c_1 = 0.0710$, $c_2 = 0.0048$, and $c_k < 10^{-3}$ for $k \geq 3$.

From c_k thus determined and I_i observed for methyl palmitate, D_j (the ^{13}C-content distribution in 16:0) is calculated by solving the set of Eqs. (6-0) to (6-16). The result is presented in Figs. 19c, e, and g.

$(M-31)^+$ and $(M-32)^+$ fragment ions of methyl palmitoleate

The ^{13}C-content distribution in 16:1 was calculated from the mass spectrum of fragment ions of methyl palmitoleate. The fragments, $(M-31)^+$ and $(M-32)^+$, have structures, $C_{15}H_{29}CO$ and $C_{15}H_{28}CO$, and contain 16 carbon atoms that can be enriched with ^{13}C. M_0 of the fragments are 237 and 236, respectively. For each of the fragments, the mathematical treatment used in the molecular ion of methyl palmitate can be applied. It is assumed that the relative yields of the fragments are β and α, that the splitting coefficients are c_k^β and c_k^α for the $(M-31)^+$ and $(M-32)^+$ fragments, respectively, and that I_i is normalized for m/e = 236 + i. Then, I_i, D_j, α, β, c_k^α and c_k^β should satisfy the following equations:

$$
\begin{aligned}
I_0 &= \alpha(c_0^\alpha D_0) \\
I_1 &= \alpha(c_1^\alpha D_0 + c_0^\alpha D_1) + \beta(c_0^\beta D_0) \\
&\cdots\cdots\cdots\cdots\cdots \\
I_{16} &= \alpha(c_{16}^\alpha D_0 + \cdots\cdots + c_0^\alpha D_{16}) + \beta(c_{15}^\beta D_0 + \cdots\cdots + c_0^\beta D_{15})
\end{aligned}
\qquad \text{----------(9)}
$$

These equations can be written in the same form as Eqs. (6-0) to (6-16) if M_0 is set 236 and c_k is defined as follows:

$$
\begin{aligned}
c_0 &= \alpha c_0^\alpha \\
c_1 &= \alpha c_1^\alpha + \beta c_0^\beta \\
&\cdots\cdots\cdots\cdots\cdots
\end{aligned}
$$

$$c_k = \alpha c_k^{\alpha} + \beta c_{k-1}^{\beta} \qquad \text{----------(10)}$$

.....

$$c_{16} = \alpha c_{16}^{\alpha} + \beta c_{15}^{\beta}$$

c_k is determined from I_i in the mass spectrum for the $(M-31)^+$ and $(M-32)^+$ fragments of unenriched methyl palmitoleate (Fig. 18b) and D_j for the unenriched 16:1 which is the same as D_j for the unenriched 16:0 (Eq. (8)). The observed values for I_i (Fig. 18b) were:

$I_0 = 0.6047$, $I_1 = 0.3278$, $I_2 = 0.0496$, $I_3 = 0.0151$, $I_4 = 0.0028$ and $I_i < 10^{-3}$ for $i \geq 5$.

With these values, c_k is determined by solving Eqs. (6-0) to (6-4):

$c_0 = 0.7224$, $c_1 = 0.2622$, $c_2 = 0.0014$, $c_3 = 0.0132$ and $c_k < 10^{-3}$ for $k \geq 4$.

From c_k thus determined and I_i observed for methyl palmitoleate, D_j (the ^{13}C-content distribution in 16:1) is calculated by solving the set of Eqs. (6-0) to (6-16). The result is presented in Figs. 19d, f and h.

$(M-90)^+$ and $(M-91)^+$ fragment ions of tris(TMS)glycerol

The ^{13}C-content distribution in glycerol was calculated from the mass spectrum of fragment ions of tris(TMS)glycerol. The fragment ions $(M-90)^+$ and $(M-91)^+$ have structures, $C_3H_5(O\text{-}TMS)_2$ and $C_3H_4(O\text{-}TMS)_2$, and contain 3 carbon atoms that can be enriched with ^{13}C. M_0 of the fragments $(M-90)^+$ and $(M-91)^+$ are 218 and 217, respectively. I_i is

normalized for m/e = 217+i, and the same treatment as in methyl palmitoleate is applied. Then, I_i, D_j and c_k should satisfy a set of Eqs. (6-0) to (6-3). Since I_4, I_5 and I_6 were not negligible, Eqs. (6-4) to (6-6) were also used to accurately determine c_k.

c_k is determined from I_i for $(M-90)^+$ and $(M-91)^+$ fragments of unenriched tris(TMS)glycerol (Fig. 20) and D_j for the unenriched Gly. The observed values for I_i were:

$I_0 = 0.0709$, $I_1 = 0.6954$, $I_2 = 0.1419$, $I_3 = 0.0612$,
$I_4 = 0.0236$, $I_5 = 0.0049$, $I_6 = 0.0021$.

D_j is calculated according to the following equation:

$$D_j = \binom{3}{j} \cdot (0.01107)^j \cdot (0.98893)^{3-j} \qquad \text{----------(11)}$$

Then,

$D_0 = 0.9672$, $D_j = 0.0325$, $D_2 = 0.0003$, $D_3 < 10^{-4}$.

With these values, c_k is determined for the case of unenriched Gly by solving Eqs. (6-0) to (6-6).

$C_0 = 0.0733$, $c_1 = 0.7165$, $c_2 = 0.1226$, $c_3 = 0.0589$,
($c_4 = 0.0224$, $c_5 = 0.0043$, $c_6 = 0.0020$)

From c_k (k= 0, 1, 2, 3) thus determined and I_i observed for tris(TMS)glycerol, D_j (the ^{13}C-content distribution in Gly) is calculated by solving the

set of Eqs. (6-0) to (6-3). The result is presented in Figs. 21b-g.

VIII. APPENDIX 2 Calculation of the ^{13}C-content distributions of the isotope combinations of Gly-C_{16}

The ^{13}C-content distribution in Gly-C_{16} (D_i^{GP} , i= 0,1,...,19) is given by the convolution of those in palmitic or palmitoleic acid (D_i^P , i= 0,1,...,16) and glycerol (D_i^G , i= 0,1,2,3):

$$D_i^{GP} = \sum_{j=0}^{16} D_j^P \cdot D_{i-j}^G \quad (\text{for } 3 \geq i-j \geq 0) \qquad \text{----------(12)}$$

An example of the result is presented in Fig. 22.

Chapter 6 Discussion

Mechanism for the lipid biosynthesis

The biosynthetic pathways of lipids and lipid molecular species in Anabaena variabilis are elucidated in the present study. The result in tracer experiments with ^{14}C in Chapter 2 demonstrates that GlcDG, PG and SqDG are synthesized de novo. GlcDG is converted to GalDG by epimerization of the glucose unit, and a part of GalDG is further converted to Gal_2DG by galactosylation. The mechanism for the biosynthesis of GalDG in the blue-green alga is different from either of the two mechanisms in higher plants, in which GalDG is produced by galactosylation of DG [68] and by replacement of the phosphocholine group of PC by galactose [51, 55]. The mechanism for the biosynthesis of Gal_2DG in the blue-green alga is similar to one of the two mechanisms in higher plants [68, 69]. The other mechanism which involves a dismutation of two GalDG molecules [70] does not seem active in the blue-green alga.

The result in tracer experiments with ^{14}C in Chapter 3 demonstrates that the 18:0/16:0 molecular species are the primary products of lipid biosynthesis and that they are rapidly converted to 18:1/16:0 and then to more unsaturated molecular species. The fact that the intramolecular distribution of radioactivity in the lipid molecules (Chapters 2 and 3) remains almost constant during the desaturation suggests a possibility that 16:0, 18:0, 18:1 and 18:2 are directly desaturated in the complex lipids.

Two mechanisms are known in the biosynthesis of unsaturated fatty acids. They are termed "anaerobic pathway" and "aerobic desaturation". The anaerobic pathway is operative in certain bacteria like Escherichia coli [106, 107, 110] and Brevibacterium ammoniagenes [123]. The aerobic

desaturation of acyl-CoA is demonstrated in a wide variety of organisms such as Tetrahymena [141], yeasts [151, 153], fungus [155] and animals [167, 169]. The aerobic desaturation of 18:0-ACP is demonstrated in higher plants [54] and Euglena gracilis [48-50]. The aerobic desaturation of 18:1 group in PC is found in Tetrahymena [145], yeast [151, 152, 154], fungi[155, 156] and higher plants [64, 65]. A previous study showed that, in A. variabilis, the desaturation of 16:0 in GalDG, which is now demonstrated to be a direct one, and that of C_{18} acids in GalDG require molecular oxygen [170]. Therefore the desaturation reactions in A. variabilis are classified in the aerobic desaturation. Whether the substrates for the desaturation of C_{18} acids are complex lipids is not established yet.

It is difficult to demonstrate the occurrence of the direct desaturation of fatty acids in vivo. Tracer experiments in vivo with radioisotopes [61,63,55,15,30] could provide indecisive evidences. In vitro tracer experiments with radioactive PC demonstrate the occurrence of the direct desaturation of PC-bound fatty acids in the microsomal fractions of Tetrahymena [145], fungus [155] and yeast[151, 152, 154]. These studies, however, provide a precursor-product relationship between the substrate and the desaturated product. The lack of phospholipase activity in the microsomal fractions [152] is considered as an evidence against the possibility that the deacylation-desaturation-reacylation cycle is involved in the desaturation of lipid-bound fatty acids.

In contrast to these methods, the "isotope combination analysis" used in the present study definitely proves the occurrence of the direct desaturation of lipid-bound fatty acids in vivo. This method provides not only the precursor-product relationship, but also the absence of

intermolecular exchange of acyl groups. In this respect, it provides an information qualitatively different from that obtained in the experiments with radioactive tracers.

Regulation of biosynthesis of fatty acids and lipid molecular species by temperature

Almost all the poikilothermic organisms change the degree of unsaturation of fatty acids in response to temperature. As mentioned in Chapter 1, the temperature-acclimative mechanisms are different according to the biosynthetic pathway of unsaturated fatty acids. In the organisms in which the unsaturated fatty acids are synthesized by the anaerobic pathway, de novo synthesis of saturated and unsaturated fatty acids is modified by the temperature [110,116,118,123]. In the organisms in which the unsaturated fatty acids are synthesized by the aerobic desaturation of acyl thioesters, temperature regulates the activity of this desaturation [142, 150, 153] so that a greater amount of unsaturated fatty acids is esterified to complex lipids at lower temperatures. In contrast, in the organisms in which the unsaturated fatty acids are synthesized by the aerobic desaturation of complex lipids, the fatty acids in the preexisting lipids are directly desaturated in response to the temperature. This is the most efficient way in the temperature-acclimative change in the fatty acid unsaturation. The direct desaturation of fatty acids in the rapid response to the downward temperature shift is demonstrated for the first time in the present study.

It is shown in Chapter 4, that the activity to desaturate 16:0 to 16:1 in GalDG increases for 3 h after the downward temperature shift, and decreases afterwards. Similar transient increases in activity to produce

unsaturated fatty acids have been demonstrated in several organisms which are different in the biosynthetic pathway of unsaturated fatty acids. Escherichia coli synthesizes a greater amount of 18:1 during the first 5 h after the temperature shift from 40 to 10°C than during the isothermal growth at 10°C (hyper-response) [118]. The activity of Bacillus megaterium to desaturate exogenously added 16:0 (hyperinduction) [125, 127], and that of microsomes of Tetrahymena to desaturate 16:0-CoA [142, 143] temporarily increase after the downward temperature shift. Since these accelerated productions of unsaturated fatty acids after the downward temperature shift rapidly increase the content of unsaturated fatty acids, they serve also as rapid responses to the abrupt change in environmental temperature.

Summary

The mechanism for the lipid biosynthesis is studied in the blue-green alga (cyanobacterium), Anabaena variabilis.

In Chapter 1, previous studies on the lipid biosynthesis in the blue-green algae, photosynthetic eukaryotes, and non-photosynthetic microorganisms are reviewed. Studies on the temperature-dependent changes in the compositions of fatty acids and lipid molecular species are also described.

In Chapter 2, biosynthesis of the glycolipids is studied by tracer experiments using ^{14}C. It is suggested that monoglucosyl diacylglycerol is the primary product among glycolipids, and that the lipid is converted by epimerization of the glucose unit to monogalactosyl diacylglycerol, a part of which is, in turn, further converted to digalactosyl diacylglycerol by galactosylation.

In Chapter 3, biosynthesis of the fatty acids and lipid molecular species are studied by tracer experiments using ^{14}C. The experimental results suggest that the primary products are stearoyl-palmitoyl molecular species which are converted to oleoyl-palmitoyl and then to more unsaturated molecular species. It is also demonstrated that the biosynthetic pathways of lipid molecular species are different between the cells which are grown at 38 and 22°C.

In Chapter 4, an effect of a downward temperature shift from 38 to 22°C on the biosynthesis of lipid molecular species is studied by labeling with ^{14}C. It is suggested that the desaturations of C_{18} acids at the C-1 position of lipids are monotonously accelerated after the temperature shift, whereas the desaturation of palmitic acid in

oleoyl-palmitoyl molecular species of monogalactosyl diacylglycerol is transiently accelerated and then suppressed.

In Chapter 5, the mechanism for the desaturation of palmitic acid at the C-2 position of monogalactosyl diacylglycerol during the isothermal growth and after the downward temperature shift is studied by "isotope combination analysis", which involves labeling of the lipid with ^{13}C and mass spectrometric analysis. The experimental results definitely prove that the palmitic acid is directly desaturated; in other words, the fatty acid remains esterified to monogalactosyl diacylglycerol when it is desaturated.

In Chapter 6, the biosynthesis of lipid molecular species in A. variabilis is discussed in comparison with that in other microorganisms and higher plants. The unique mechanism for the temperature-acclimative regulation of lipid milecular species is described.

References

1. Fritsch, F. E. (1945)
"The Structure and Reproduction of the Algae", vol. 2, pp. 768-898
Cambridge Univ. Press, London

2. Fogg, G. E., Stewart, W. D. P., Fay, P. and Walsby, A. E. (1973)
"The blue-green Algae", Academic Press, London

3. Wolk, C. P. (1973)
"Physiology and cytological chemistry of blue-green algae",
Bacteriol. Rev., 37, 32-101

4. Stanier, R. Y. and Cohen-Bazire, G. (1977)
"Phototrophic prokaryotes: The cyanobacteria",
Annu. Rev. Microbiol., 31, 225-274

5. Stanier, R. Y., Kunisawa, R., Mandel, M. and Cohen-Bazire, G.
(1971) "Purification and properties of unicellular blue-green
algae (order Chroococcales)", Bacteriol. Rev., 35, 171-205

6. Waterbury, J. B. and Stanier, R. Y. (1978)
"Patterns of growth and development in pleurocapsalean cyano-
bacteria", Microbiol. Rev., 42, 2-44

7. Ono, T. and Murata, N. (1977)
"Temperature dependence of the delayed fluorescence of chloro-
phyll a in blue-green algae", Biochim. Biophys. Acta, 460,
220-229

8. Sato, N., Murata, N., Miura, Y. and Ueta, N. (1979)
"Effect of growth temperature on lipid and fatty acid compositions
in the blue-green algae, Anabaena variabilis and Anacystis
nidulans", Biochim. Biophys. Acta, 572, 19-28

9. Nichols, B. W., Harris, R. V. and James, A. T. (1965) "The lipid metabolism of blue-green algae", Biochem. Biophys. Res. Cummun., 20, 256-262

10. Nichols, B. W. and Wood, B. J. B. (1968) "The occurrence and biosynthesis of gamma-linolenic acid in a blue-green alga, Spirulina platensis" , Lipids, 3, 46-50

11. Feige, G. B., Heinz, E., Wrage, K., Cochems, N. and Ponzelar, E. (1980) "Discovery of a new glyceroglycolipid in blue-green algae and its role in galactolipid biosynthesis", in [40], pp.135-140

12. Zepke, H. D., Heinz, E., Radunz, A., Linscheid, M. and Pesch, R. (1978) "Combination and positional distribution of fatty acids in lipids from blue-green algae", Arch. Microbiol., 119, 157-162

13. Douce, R., Holtz, R. B. and Benson, A. A. (1973) "Isolation and properties of the envelope of spinach chloroplasts", J. Biol. Chem., 248, 7215-7222

14. Mackender, R. O. and Leech, R. M. (1974) "The galactolipid, phospholipid, and fatty acid composition of the chloroplast envelope membranes of Vicia faba L." , Plant Physiol., 53, 496-502

15. Appleby, R. S., Safford, R. and Nichols, B. W. (1971) "The involvement of lecithin and monogalactosyl diglyceride in linoleate synthesis by green and blue-green algae", Biochim. Biophys. Acta, 248, 205-211

16. Holton, R. W., Blecker, H. H. and Stevens, T. S. (1968) "Fatty acids in blue-green algae: possible relation to phylogenetic position", Science, 160, 545-547

17. Kenyon, C. N. (1972)
"Fatty acid composition of unicellular strains of blue-green algae", J. Bacteriol., 109, 827-834

18. Kenyon, C. N., Rippka, R. and Stanier, R. Y. (1972)
"Fatty acid composition and physiological properties of some filamentous blue-green algae", Arch. Mikrobiol., 83, 216-236

19. Kenyon, C. N. and Stanier, R. Y. (1970)
"Possible evolutionary significance of polyunsaturated fatty acids in blue-green algae", Nature, 227, 1164-1166

20. Parker, P. L., Van Baalen, C. and Maurer, L. (1967)
"Fatty acids in eleven species of blue-green algae: geochemical significance", Science, 155, 707-708

21. Holton, R. W., Blecker, H. H. and Onore, M. (1964)
"Effect of growth temperature on the fatty acid composition of a blue-green alga", Phytochemistry, 3, 595-602

22. Sastry, P. S. (1974)
"Glycosyl glycerides", Adv. Lipid Res., 12, 251-310

23. Fork, D. C., Murata, N. and Sato, N. (1979)
"Effect of growth temperature on the lipid and fatty acid composition, and the dependence on temperature of light-induced redox reactions of cytochrome f and of light energy redistribution in the thermophilic blue-green alga Synecococcus lividus"
Plant Physiol. 63, 524-530

24. Fischer, W., Heinz, E. and Zeus, M. (1973)
"The suitability of lipase from Rhizopus arrhizus delemar for analysis of fatty acid distribution in dihexosyl diglycerides, phospholipids and plant sulfolipids", Hoppe-Seyler's Z. Physiol. Chem., 354, 1115-1123

25. Safford, R. and Nichols, B. W. (1970)
"Positional distribution of fatty acids in monogalactosyl diglyceride fractions from leaves and algae. Structural and metabolic studies", Biochim. Biophys. Acta, 210, 57-64

26. Auling, G., Heinz, E. and Tulloch, A. P. (1971)
"Combination and positional distribution of fatty acids in plant galactolipids", Hoppe-Seyler's Z. Physiol. Chem., 352, 905-912

27. Tulloch, A. P., Heinz, E. and Fischer, W. (1973)
"Combination and positional distribution of fatty acids in plant sulfolipids", Hoppe-Seyler's Z. Physiol. Chem., 354, 879-889

28. Sato, N. and Murata, N. (1980)
"Temperature shift-induced responses in lipids in the blue-green alga, Anabaena variabilis. The central role of diacylmonogalactosylglycerol in thermo-adaptation", Biochim. Biophys. Acta, 619, 353-366

29. Nishihara, M., Yokota, K. and Kito, M. (1980)
"Lipid molecular species composition of thylakoid membranes", Biochim. Biophys. Acta, 617, 12-19

30. Nichols, B. W. (1968)
"Fatty acid metabolism in the chloroplast lipids of green and blue-green algae", Lipids, 3, 354-360

31. Feige, G. B. (1978)
"Physiologische Characteristika der Heterocystendifferenzierung in verschiedenen Blaualgenflechten", Ber. Deutsch. Bot. Ges., 91, 595-602

32. Shaw, R. (1966)
"The polyunsaturated fatty acids of microorganisms", Adv. Lipid Res., 4, 107-174

33. Nichols, B. W. and James, A. T. (1968)
"Acyl lipids and fatty acids of photosynthetic tissue" in "Progress in phytochemistry" (Reinhold, L. and Liwschitz, Y, eds.), Interscience, London

34. Kates, M. (1970)
"Plant phospholipids and glycolipids", Adv. Lipid Res., 8, 225-265

35. Quinn, P. J. and Williams, W. P. (1978)
"Plant lipids and their role in membrane function", Prog. Biophys. Molec. Biol., 34, 109-173

36. Hitchcock, C. and Nichols, B. W. (1971)
"Plant Lipid Biochemistry", Academic Press, London

37. Galliard, T. and Mercer, E. I. (1975)
"Recent Advances in the Chemistry and Biochemistry of Plant Lipids", Academic Press, London

38. Tevini, M. and Lichtenthaler, H. K. (1977)
"Lipids and Lipid Polymers in Higher Plants" , Springer, Berlin

39. Appelqvist, L-A. and Liljenberg, C. (1979)
"Advances in the Biochemistry and Physiology of Plant Lipids", Elsevier, Amsterdam

40. Mazliak, P., Benveniste, P., Costes, C. and Douce, R. (1980)
"Biogenesis and Function of Plant Lipids", Elsevier, Amsterdam

41. Mazliak, P. (1973)
"Lipid metabolism in plants", Annu. Rev. Plant Physiol., 24, 287-310

42. Harris, R. V., Harris, P. and James, A. T. (1965)
"The fatty acid metabolism of Chlorella vulgaris", Biochim. Biophys. Acta, 106, 465-473

43. Nichols, B. W., James, A. T. and Breuer, J. (1967)
"Interrelationships between fatty acid biosynthesis and acyl-lipid synthesis in Chlorella vulgaris" , Biochem. J., 104, 486-496

44. Gurr, M. I., Robinson, M. P. and James, A. T. (1969)
"The mechanism of formation of polyunsaturated fatty acids by photosynthetic tissue. The tight coupling of oleate desaturation with phospholipid synthesis in Chlorella vulgaris", Eur. J. Biochem., 9, 70-78

45. Gurr, M. I. and Brawn, P. (1970)
"The biosynthesis of polyunsaturated fatty acids by photo-synthetic tissue. The composition of phosphatidyl choline species in Chlorella vulgaris during the formation of linoleic acid", Eur. J. Biochem., 17, 19-22

46. Gurr, M. I. (1971)
"The biosynthesis of polyunsaturated fatty acids in plants" Lipids, 6, 266-273

47. Nichols, B. W. and Moorhouse, R. (1969)
"The separation, structure and metabolism of monogalactosyl diglyceride species in Chlorella vulgaris", Lipids, 4, 311-316

48. Nagai, J. and Bloch, K. (1965)
"Synthesis of oleic acid by Euglena gracilis", J. Biol. Chem., 240, PC3702-PC3703

49. Nagai, J. and Bloch, K.(1966)
"Enzymatic desaturation of stearyl acyl carrier protein", J. Biol. Chem., 241, 1925-1927

50. Nagai, J. and Bloch, K.(1968)
"Enzymatic desaturation of stearyl acyl carrier protein", J. Biol. Chem., 243, 4626-4633

51. Roughan, P. G., Holland, R. and Slack, C. R. (1980)
"The role of chloroplast and microsomal fractions in polar-lipid synthesis from [1-^{14}C] acetate by cell-free preparations from spinach (Spinacia oleracea) leaves", Biochem. J., 188, 17-24

52. Brooks, J. L. and Stumpf, P. K. (1965)
"A soluble fatty acid synthesizing system from lettuce chloroplasts", Biochim. Biophys. Acta, 98, 213-216

53. Jaworski, J. G., Goldschmidt, E. E. and Stumpf, P. K. (1974)
"Fat metabolism in higher plants. Properties of the palmityl acyl carrier protein: stearyl acyl carrier protein elongation system in maturing safflower seed extracts", Arch. Biochem. Biophys., 163, 769-776

54. Jaworski, J. G. and Stumpf, P. K. (1974)
"Fat metabolism in higher plants. Properties of a soluble stearyl-acyl carrier protein desaturases from maturing Carthamus tinctorius", Arch. Biochem. Biophys., 162, 158-165

55. Slack, C. R., Roughan, P. G. and Balasingham, N. (1977)
"Labelling studies in vivo on the metabolism of the acyl and glycerol moieties of the glycerolipids in the developing maize leaf", Biochem. J., 162, 289-296

56. Williams, J. P., Watson, G. R. and Leung, S. P. K. (1976) "Galactolipid synthesis in Vicia faba leaves. II. Formation and desaturation of long chain fatty acids in phosphatidylcholine, phosphatidylglycerol and the galactolipids", Plant Physiol., 57, 179-184

57. Simpson, E. E. and Williams, J. P. (1979) "Galactolipid synthesis in Vicia faba leaves. IV. Site(s) of fatty acid incorporation into the major glycerolipids", Plant Physiol., 63, 674-676

58. Williams, J. P., Watson, G. R., Khan, M-U. and Leung, S. (1975) "Galactolipid synthesis in Vicia faba leaves. I. Galactose, glycerol, and fatty acid labeling after $^{14}CO_2$ feeding", Plant Physiol., 55, 1038-1042

59. Williams, J. P., Simpson, E. E. and Chapman, D. J. (1979) "Galactolipid synthesis in Vicia faba leaves. III. Site(s) of galactosyl transferase activity", Plant Physiol., 63, 669-673

60. Williams, J. P. (1980) "Galactolipid synthesis in Vicia faba leaves. V. Redistribution of ^{14}C-labelling in the polar moieties and the ^{14}C-labelling kinetics of the fatty acids of the molecular species of mono- and digalactosyl diacylglycerols", Biochim. Biophys. Acta, 618, 461-472

61. Siebertz, H. P. and Heinz, E. (1977) "Labelling experiments on the origin of hexa- and octadecatrienoic acids in galactolipids from leaves", Z. Naturforsch. C. 32, 193-205

62. Joyard, J., Douce, R., Siebertz, H. P. and Heinz, E. (1980) "Distribution of radioactive lipids between envelopes and thylakoids from chloroplasts labelled in vivo", Eur. J. Biochem., 108, 171-176

63. Siebertz, H. P., Heinz, E., Joyard, J. and Douce, R. (1980) "Labelling in vivo and in vitro of molecular species of lipids from chloroplast envelopes and thylakoids", Eur. J. Biochem., 108, 177-185

64. Slack, C. R., Roughan, P. G. and Terpstra, J. (1976) "Some properties of a microsomal oleate desaturase from leaves", Biochem. J., 155, 71-80

65. Stymne, S. and Appelqvist, L-A. (1978) "The biosynthesis of linoleate from oleoyl-CoA via oleoyl-phosphatidylcholine in microsomes of developing safflower seeds", Eur. J. Biochem., 90, 223-229

66. Stymne, S. and Appelqvist, L-A. (1980) "The biosynthesis of linoleate and α-linolenate in homogenates from developing soya bean cotyledons", Plant Sci. Lett., 17, 287-294

67. Joyard, J. and Douce, R. (1976) "Mise en évidence et rôle des diacylglycérols de l'enveloppe des chloroplastes d'épinard", Biochim. Biophys. Acta, 424, 125-131

68. Ongun, A. and Mudd, J. B. (1968) "Biosynthesis of galactolipids in plants", J. Biol. Chem., 243, 1558-1566

69. Siebertz, M. and Heinz, E. (1977)
"Galactosylation of different monogalactosyldiacylglycerols by cell-free preparations from pea leaves", Hoppe-Seyler's Z. Physiol. Chem., 358, 27-34

70. Van Besouw, A. and Wintermans, J. F. G. M. (1978)
"Galactolipid formation in chloroplast envelopes. I. Evidence for two mechanisms in galactosylation", Biochim. Biophys. Acta, 529, 44-53

71. Brock, T. D. (1978)
"Thermophilic Microorganisms and Life at High Temperatures", Springer, New York

72. Berry, J. and Björkman, O. (1980)
"Photosynthetic response and adaptation to temperature in higher plants", Annu. Rev. Plant Physiol., 31, 491-543

73. Quinn, P. J. (1976)
"The Molecular Biology of Cell Membranes", Macmillan Press, London

74. Melchior, D. L. and Steim, J. M. (1976)
"Thermotropic transitions in biomembranes", Annu. Rev. Biophys. Bioeng., 5, 205-238

75. Chapman, D. (1969)
"Physical studies of lipid-lipid and lipid-protein interactions", Lipids, 4, 251-260

76. Chapman, D., Urbina, J. and Keough, K. M. (1974)
"Biomembrane phase transitions: Studies of lipid-water systems using differential scanning calorimetry", J. Biol. Chem., 249, 2512-2521

77. Träuble, H. (1971)
"Phasenumwandlungen in Lipiden. Mögliche Schaltprozesse in biologischen Membranen", Naturwissenschaften, 58, 277-284

78. Gulik-Krzywicki, T., Rivas, E. and Luzzati, V. (1967)
"Structure et polymorphisme des lipides: Étude par diffraction des rayons X du système formé de lipides de mitochondries de coeur de boeuf et d'eau", J. Mol. Biol., 27, 303-322

79. Cullis, P. R. and De Kruijff, B. (1979)
"Lipid polymorphism and the functional roles of lipids in biological membranes", Biochim. Biophys. Acta, 559, 399-420

80. Janoff, A. S., Haug, A. and McGroarty, E. J. (1979)
"Relationship of growth temperature and thermotrophic lipid phase changes in cytoplasmic and outer membranes from Escherichia coli K 12", Biochim. Biophys. Acta, 555, 56-66

81. Nakayama, H., Mitsui, T., Nishihara, M. and Kito, M. (1980)
"Relation between growth temperature of E. coli and phase transition temperatures of its cytoplasmic and outer membranes", Biochim. Biophys. Acta, 601, 1-10

82. Furtado, D., Williams, W. P., Brain, A. P. R. and Quinn, P. J. (1979) "Phase separations in membranes of Anacystis nidulans grown at different temperatures", Biochim. Biophys. Acta, 555, 352-357

83. Tsukamoto, Y., Ueki, T., Mitsui, T., Ono, T. and Murata, N. (1980) "Relationship between growth temperature of Anacystis nidulans and phase transition temperature of its thylakoid membranes", Biochim. Biophys. Acta, 602, 673-675

84. Kimelberg, H. K. and Papahadjopoulos, D. (1972) "Phospholipid requirements for (Na+K)-ATPase activity: Head-group specificity and fatty acid fluidity", Biochim. Biophys. Acta, 282, 277-292

85. Kimelberg, H. K. and Papahadjopoulos, D. (1974) "Effects of phospholipid acyl chain fluidity, phase transition, and cholesterol on (Na+K)-stimulated adenosine triphosphatase", J. Biol. Chem., 249, 1071-1080

86. Heller, K. B. and Hoeffer, M. (1978) "Temperature dependence of the apparent affinity and the maximum velocity of the membrane-bound monosaccharide transport system in the yeast Rhodotorula gracilis", Biochim. Biophys. Acta, 514, 172-177

87. Silbert, D. F. (1975) "Genetic modification of membrane lipid", Annu. Rev. Biochem., 44, 315-339

88. Cronan, J. E. Jr. and Gelmann, E. P. (1975) "Physical properties of membrane lipids: biological relevance and regulation", Bacteriol. Rev., 39, 232-256

89. Haest, C. W. M., De Gier, J., Van Es, G. A., Verkleij, A. J. and Van Deenen, L. L. M. (1972) "Fragility of the permeability barrier of Escherichia coli", Biochim. Biophys. Acta, 288, 43-53

90. Leder, I. G. (1972) "Interrelated effects of cold shock and osmotic pressure on the permeability of Escherichia coli", J. Bacteriol., 111, 211-219

91. Akutsu, H., Akamatsu, Y., Sinbo, T, Uehara, K., Takahashi, K. and Kyogoku, Y. (1980) "Evidence for phase separation in the membrane of an osmotically stabilized fatty acid auxotroph of E. coli and its biological significance", Biochim. Biophys. Acta, 598, 437-446

92. Murata, N., Troughton, J. H. and Fork, D. C. (1975) "Relationships between the transition of the physical phase of membrane lipids and photosynthetic parameters in Anacystis nidulans and lettuce and spinach chloroplasts", Plant Physiol., 56, 508-517

93. Murata, N., Ono, T. and Sato, N. (1979) "Lipid phase of membrane and chilling injury in the blue-green alga", In "Low Temperature Stress in Crop Plants" (Lyons, J. M., Graham, D. and Raison, J. K. eds.), Academic Press, New York pp. 337-345

94. Murata, N. and Fork, D. C. (1975) "Temperature dependence of chlorophyll a fluorescence in relation to the physical phase of membrane lipids in algae and higher plants", Plant Physiol., 56, 791-796

95. Ono, T. and Murata, N. (1979) "Temperature dependence of the photosynthetic activities in the thylakoid membranes from the blue-green alga, Anacystis nidulans", Biochim. Biophys. Acta, 545, 69-76

96. Sato, N. and Murata, N. (1980) "Desaturation of fatty acids in lipids in response to the growth temperature in the blue-green alga, Anabaena variabilis" , In [40], pp. 207-210

97. Kleinschmidt, M. G. and McMahon, V. A. (1970)
"Effect of growth temperature on the lipid composition of Cyanidium caldarium. II. Glycolipid and phospholipid components", Plant Physiol., 46, 290-293

98. Adams, B. L., McMahon, V. A. and Seckbach, J. (1971)
"Fatty acids in the thermophilic alga, Cyanidium caldarium" , Biochem. Biophys. Res. Commun., 42, 359-365

99. Bedord, C. J., McMahon, V. A. and Adams, B. L. (1978)
"α-Linolenic acid biosynthesis in Cyanidium caldarium" , Arch. Biochem. Biophys., 185, 15-20

100. Patterson, G. W. (1970)
"Effect of culture temperature on fatty acid composition of Chlorella sorokiniana", Lipids, 5, 597-600

101. Blee, E. and Schantz, R. (1978)
"Biosynthesis of galactolipids in Euglena gracilis. II. Changes in fatty acid composition of galactolipids during chloroplast development", Plant Sci. Lett., 13, 257-267

102. Lem, N. W., Khan, M., Watson, G. R. and Williams, J. P. (1980)
"The effect of light intensity, day length, and temperature on fatty acid synthesis and desaturation in Vicia faba L." , J. Exp. Bot., 31, 289-298

103. Slack, C. R. and Roughan, P. G. (1978)
"Rapid temperature-induced changes in the fatty acid composition of certain lipids in developing linseed and soya-bean cotyledons", Biochem. J., 170, 437-439

104. Rivera, C. M. and Penner, D. (1978)
"Rapid changes in soybean root membrane lipids with altered temperature", Phytochemistry, 17, 1269-1272

105. Raetz, C. R. H. (1978)
"Enzymology, genetics, and regulation of membrane phospholipid synthesis in Escherichia coli", Microbiol. Rev., 42, 614-659

106. Volpe, J. J. and Vagelos, P. R. (1973)
"Saturated fatty acid biosynthesis and its regulation", Annu. Rev. Biochem.,42, 21-60

107. Bloch, K. and Vance, D. (1977)
"Control mechanisms in the synthesis of saturated fatty acids", Annu. Rev. Biochem., 46, 263-298

108. Vagelos, P. R., Alberts, A. W. and Majerus, P. W. (1969)
"Mechanism of saturated fatty acid biosynthesis in Escherichia coli", Methods Enzymol., 14, 39-43

109. Rosenfeld, I. S., D'Agnolo, G. and Vagelos, P. R. (1973)
"Synthesis of unsaturated fatty acids and the lesion in fab B mutants", J. Biol. Chem., 248, 2452-2460

110. Garwin, J. L., Klages, A. L. and Cronan, J. E. Jr. (1980)
"β-Ketoacyl-acyl carrier protein synthase II of Escherichia coli. Evidence for function in the thermal regulation of fatty acid synthesis", J. Biol. Chem., 255, 3263-3265

111. Cronan, J. E. Jr., Reed, R., Taylor, F. R. and Jackson, M. B. (1979) "Properties and biosynthesis of cyclopropane fatty acids in Escherichia coli" , J. Bacteriol., 138, 118-121

112. Ishinaga, M., Nishihara, M. and Kito, M. (1976) "Purification and positional specificity of sn-glycerol-3-phosphate acyltransferase from Escherichia coli membranes", Biochim. Biophys. Acta, 450, 269-272

113. Okuyama, H. and Wakil, S. J. (1973) "Positional specificities of acyl coenzyme A: glycerophosphate and acyl coenzyme A: monoacylglycerophosphate acyltransferases in Escherichia coli", J. Biol. Chem., 248, 5197-5205

114. Marr, A. G. and Ingraham, J. L. (1962) "Effect of temperature on the composition of fatty acids in Escherichia coli", J. Bacteriol., 84, 1260-1267

115. Kito, M., Aibara, S., Kato, M. and Hata, T. (1972) "Differences in fatty acid composition among phosphatidylethanolamine, phosphatidylglycerol and cardiolipin of Escherichia coli", Biochim. Biophys. Acta, 260, 475-478

116. Nishihara, M., Ishinaga, M., Kato, M. and Kito, M. (1976) "Temperature-sensitive formation of the phospholipid molecular species in Escherichia coli membranes", Biochim. Biophys. Acta, 431, 54-61

117. Ishinaga, M., Kanamoto, R. and Kito, M. (1979) "Distribution of phospholipid molecular species in outer and cytoplasmic membranes of Escherichia coli" , J. Biochem., 86, 161-165

118. Okuyama, H., Yamada, K., Kameyama, Y., Ikezawa, H., Akamatsu, Y. and Nojima, S. (1977) "Regulation of membrane lipid synthesis in Escherichia coli", Biochemistry, 16, 2668-2673

119. Cronan, J. E. Jr. (1975)
"Thermal regulation of the membrane lipid composition of Escherichia coli", J. Biol. Chem., 250, 7074-7077

120. Sinensky, M. (1974)
"Homeoviscous adaptation —— A homeostatic process that regulates the viscosity of membrane lipids in Escherichia coli", Proc. Nat. Acad. Sci. USA., 71, 522-525

121. Fung, L. W-M., Pratt, E. A. and Ho, C. (1979)
"Biochemical and biophysical studies on the interaction of a membrane-bound enzyme, D-lactate dehydrogenase from Escherichia coli with phospholipids", Biochemistry, 18, 317-324

122. Cronan, J. E. Jr. (1978)
"Molecular biology of bacterial membrane lipids", Annu. Rev. Biochem., 47, 163-189

123. Kawaguchi, A., Seyama, Y., Sasaki, K., Okuda, S. and Yamakawa,T. (1980) "Thermal regulation of fatty acid synthetase from Brevibacterium ammoniagenes" , J. Biochem., 85, 865-869

124. Fulco, A. J. (1970)
"The biosynthesis of unsaturated fatty acids by bacilli. II. Temperature-dependent biosynthesis of polyunsaturated fatty acids", J. Biol. Chem., 245, 2985-2990

125. Fulco, A. J. (1970)
"Temperature-mediated hyper-induction of fatty acid desaturating enzyme", Biochim. Biophys. Acta, 218, 558-560

126. Fulco, A. J. (1969)
"The biosynthesis of unsaturated fatty acids by bacilli. I. Temperature induction of the desaturation reaction", J. Biol. Chem., 244, 889-895

127. Fujii, D. K. and Fulco, A. J. (1977)
"Biosynthesis of unsaturated fatty acids by Bacilli. Hyperinduction and modulation of desaturase synthesis", J. Biol. Chem., 252, 3660-3670

128. Fulco, A. J. (1972)
"The biosynthesis of unsaturated fatty acids by Bacilli. III. Uptake and utilization of exogenous palmitate", J. Biol. Chem., 247, 3503-3510

129. Fulco, A. J. (1974)
"Metabolic alterations of fatty acids", Annu. Rev. Biochem., 43, 215-241

130. Lombardi, F. J. and Fulco, A. J. (1980)
"Temperature-mediated hyperinduction of fatty acid desaturation in pre-existing and newly formed fatty acids synthesized endogenously in Bacillus megaterium", Biochim. Biophys. Acta, 618, 359-363

131. Melchior, D. L. and Steim, J. M. (1977)
"Control of fatty acid composition of Acholeplasma laidlawii membranes", Biochim. Biophys. Acta, 466, 148-159

132. Koroly, M. J. and Conner, R. L. (1976)
"Unsaturated fatty acid biosynthesis in Tetrahymena", J. Biol. Chem., 251, 7588-7592

133. Thompson, G. A. Jr. and Nozawa, Y. (1977)
"Tetrahymena: a system for studying dynamic membrane alterations within the eukaryotic cell", Biochim. Biophys. Acta, 472, 55-92

134. Erwin, J. and Bloch, K. (1963)
"Lipid metabolism of ciliated protozoa", J. Biol. Chem., 238, 1618-1624

135. Wunderlich, F., Speth, V., Batz, W. and Kleining, H. (1973)
"Membranes of Tetrahymena. III. The effect of temperature on membrane core structures and fatty acid composition of Tetrahymena cells", Biochim. Biophys. Acta, 298, 39-49

136. Fukushima, H., Martin, C. E., Iida, H., Kitajima, Y., Thompson, G. A. Jr. and Nozawa, Y. (1976) "Changes in membrane lipid composition during temperature adaptation by a thermotolerant strain of Tetrahymena pyriformis", Biochim. Biophys. Acta, 431, 165-179

137. Nozawa, Y. and Kasai, R. (1978)
"Mechanism of thermal adaptation of membrane lipids in Tetrahymena pyriformis NT-1. Possible evidence for temperature-mediated induction of palmitoyl-CoA desaturase", Biochim. Biophys. Acta, 529, 54-66

138. Kasai, R., Kitajima, Y., Martin, C. E., Nozawa, Y., Skriver, L. and Thompson, G. A. Jr. (1976) "Molecular control of membrane properties during temperature acclimation. Membrane fluidity regulation of fatty acid desaturase action?", Biochemistry, 15, 5228-5233

139. Martin, C. E., Hiramatsu, K., Kitajima, Y., Nozawa, Y., Skriver, L. and Thompson, G. A. Jr. (1976) "Molecular control of membrane properties during temperature acclimation. Fatty acid desaturase regulation of membrane fluidity in acclimating Tetrahymena cells", Biochemistry, 15, 5218-5227

140. Skriver, L. and Thompson, G. A. Jr. (1979)
"Temperature-induced changes in fatty acid unsaturation of Tetrahymena membranes do not require induced fatty acid desaturase synthesis", Biochim. Biophys. Acta, 572, 376-381

141. Fukushima, H., Nagao, S., Okano, Y. and Nozawa, Y. (1977)
"Studies on Tetrahymena membranes. Palmitoyl-coenzyme A desaturase, a possible key enzyme for temperature adaptation in Tetrahymena microsomes", Biochim. Biophys. Acta, 488, 442-453

142. Fukushima, H., Nagao, S. and Nozawa, Y. (1979)
"Further evidence for changes in the level of palmitoyl-CoA desaturase during thermal adaptation in Tetrahymena pyriformis", Biochim. Biophys. Acta,572, 178-182

143. Kasai, R. and Nozawa, Y. (1980)
"Regulation mechanism of palmitoyl-CoA desaturase activity in thermal adaptation. Induction in non-growing Tetrahymena cells deprived of pre-existing desaturase", Biochim. Biophys. Acta, 617, 161-166

144. Nagao, S., Fukushima, H. and Nozawa, Y. (1978)
"Studies on Tetrahymena membranes. Substrates for desaturation of fatty acyl chains in Tetrahymena pyriformis microsomes", Biochim. Biophys. Acta, 530, 165-174

145. Kameyama, Y., Yoshioka, S. and Nozawa, Y. (1980)
"The occurrence of direct desaturation of phospholipid acyl chain in Tetrahymena pyriformis", Biochim. Biophys. Acta, 618, 214-222

146. Rattray, J. B. M., Schibeci, A. and Kidby, D. K. (1975) "Lipids of Yeasts", Bacteriol. Rev., 39, 197-231

147. Schweizer, E., Kniep, B., Castorph, H. and Holzner, U. (1973) "Pantetheine-free mutants of the yeast fatty-acid-synthetase complex", Eur. J. Biochem., 39, 353-362

148. Lynen, F., Hopper-Kessel, I. and Eggerer, H. (1964) "Zur Biosynthese der Fettsäuren. III. Die Fettsäuresynthetase der Hefe und die Bildung enzymgebundener Acetessigsäure", Biochem. Z., 340, 95-124

149. Brown, C. M. and Rose, A. H. (1969) "Fatty-acid composition of Candida utilis as affected by growth temperature and dissolved-oxygen tension", J. Bacteriol., 99, 371-378

150. Kates, M. and Paradis, M. (1973) "Phospholipid desaturation in Candida lipolytica as a function of temperature and growth", Can. J. Biochem., 51, 184-197

151. Pugh, E. L. and Kates, M. (1975) "Characterization of a membrane-bound phospholipid desaturase system of Candida lipolytica", Biochim. Biophys. Acta, 380, 442-453

152. Pugh, E. L. and Kates, M. (1979) "Membrane-bound phospholipid desaturases", Lipids, 14, 159-165

153. Meyer, F. and Bloch, K. (1963) "Effect of temperature on the enzymatic synthesis of unsaturated fatty acids in Torulopsis utilis", Biochim. Biophys. Acta, 77, 671-673

154. Talamo, B., Chang, N. and Bloch, K. (1973)
"Desaturation of oleyl phospholipid to linoleyl phospholipid in Torulopsis utilis", J. Biol. Chem., 248, 2738-2742

155. Baker, N. and Lynen, F.(1971)
"Factors involved in fatty acyl CoA desaturation by fungal microsomes. The relative roles of acyl CoA and phospholipids as substrates", Eur. J. Biochem., 19, 200-210

156. Wilson, A. C., Adams, W. C., Miller, R. W. (1980)
"Lipid involvement in oleoyl CoA desaturase activity of Fusarium oxysporum microsomes", Can. J. Biochem., 58, 97-102

157. Kratz, W. A. and Myers, J. (1955)
"Nutrition and growth of several blue-green algae", Am. J. Bot., 42, 282-287

158. Bligh, E. G. and Dyer, W. J. (1959)
"A rapid method of total lipid extraction and purification", Can. J. Biochem. Physiol., 37, 911-917

159. Pohl, P., Glasl, H. and Wagner, H. (1970)
"Zur Analytik pflanzlicher Glyco- und Phospholipoide und ihrer Fettsäuren. I. Eine neue dünnschichtchromatographische Methode zur Trennung pflanzlicher Lipoide und quantitativen Bestimmung ihrer Fettsäure-Zusammensetzung", J. Chromatogr., 49, 488-492

160. Lechevallier, D., Bahl, J. and Monéger, R. (1972)
"Étude microanalytique des lipides dans de petits échantillons d'étioplastes et de chloroplastes isolés de feuilles de blé", Comptes Rendus Acad. Sci. Paris, série D, 275, 963-966

161. Sweeley, C. C. and Walker, B. (1964)
"Determination of carbohydrates in glycolipides and gangliosides by gas chromatography", Anal. Chem., 36, 1461-1466

162. Omura, S. (1976)
"The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis", Bacteriol. Rev., 40, 681-697

163. Ferrari, R. A. and Benson, A. A. (1961)
"The path of carbon in photosynthesis of the lipids", Arch. Biochem. Biophys., 93, 185-192

164. Kito, M., Ishinaga, M., Nishihara, M., Kato, M., Sawada, S. and Hata, S. (1975) "Metabolism of the phosphatidylglycerol molecular species in Escherichia coli", Eur. J. Biochem., 54, 55-63

165. Kuksis, A. (1976)
"Gas chromatography of neutral acylglycerols", In "Lipid Chromatographic Analysis", 2nd ed.,(Marinetti, G. V. ed.), Marcel-Dekker, New York, vol.1, pp. 215-337

166. Itabashi, Y. and Takagi, T. (1980)
"Gas chromatographic separations of di- and monoacylglycerols based on the degree of unsaturation and positional placement of acyl groups", Lipids, 15, 205-215

167. Oshino, N., Imai, Y. and Sato, R. (1966)
"Electron-transfer mechanism associated with fatty acid desaturation catalyzed by liver microsomes", Biochim. Biophys. Acta, 128, 13-28

168. Bergman, R. G. (1980)
"Use of isotope crossover experiments in investigating carbon-carbon bond-formating reactions of binuclear dialkylcobalt complexes", Acc. Chem. Res., 13, 113-120

169. Okayasu, T., Ono, T., Shinojima, K. and Imai, Y. (1977)
"Involvement of cytochrome b_5 in the oxidative desaturation of linoleic acid to γ-linolenic acid in rat liver microsomes". Lipids, 12, 267-271

170. Sato, N. and Murata, N. (1981)
"Studies on temperature shift-induced desaturation of fatty acids in monogalactosyl diacylglycerol in the blue-green alga (cyanobacterium), Anabaena variabilis",
Plant & Cell Physiol., submitted.

171. Mackinney, G. (1941)
"Absorption of light by chlorophyll solutions".
J. Biol. Chem., 140, 315-322

参考論文

Reference papers

佐藤直樹

Naoki SATO

1. "Preparation of chlorophyll a, chlorophyll b and bacteriochlorophyll a by means of column chromatography with diethylaminoethylcellulose"
2. "Studies on the absorption spectra of chlorophyll a in aqueous dispersions of lipids from the photosynthetic membranes"
3. "Effect of growth temperature on lipid and fatty acid compositions in the blue-green algae, Anabaena variabilis and Anacystis nidulans"
4. "Lipid phase of membrane and chilling injury in the blue-green alga, Anacystis nidulans"
5. "Effect of growth temperature on the lipid and fatty acid composition, and the dependence on temperature of light-induced redox reactions of cytochrome f and of light energy redistribution in the thermophilic blue-green alga Synecococcus lividus"
6. "Temperature shift-induced responses in lipids in the blue-green alga, Anabaena variabilis. The central role of diacylmonogalactosylglycerol in thermo-adaptation"
7. "Desaturation of fatty acids in lipids in response to the growth temperature in the blue-green alga, Anabaena variabilis"

8. "Separation and characterization of thylakoid and cell envelope of the blue-green alga (cyanobacterium) Anacystis nidulans"

9. "Studies on temperature shift-induced desaturation of fatty acids in monogalactosyl diacylglycerol in the blue-green alga (cyanobacterium), Anabaena variabilis"

以上.