STUDIES ON TOXIC EFFECTS OF DIETARY FATTY ACIDS ON CHINESE HAMSTER V79 CELLS

REIKO URADE

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CONTENTS

ABBREVIATIONS

PPO	2,5-diphenyloxazole
POPOP	1,4-bis(2-(5-phenyloxazolyl))benzene
Tris	tris(hydroxymethyl)aminomethane
EDTA	ethylenediaminetetraacetic acid disodium salt

I GENERAL INTRODUCTION

Protein, carbohydrate and lipid in foods are essential nutritional components for animals. However, they show toxic effects on animals under special conditions. Intake of excess amounts of edible lipids causes several geriatric disorders. Saturated fatty acids are known to be one of causes atherosclerosis and cardiopathy (1), whereas unsaturated fatty acids influence cellular immune responces (2, 3) and increase tumor incidence (4). However, mechanism of these toxic effects of lipids still remain unclear due to the difficulties in experimental techniques.

Lipids are transported to individual organ cell through blood-flow after digestion and absorption, and then fatty acids released from lipids are incorporated into cells. A part of them are oxidized to produce energy in mitochondria and the rest are used to the synthesis of phospholipid and triacylglycerol in endoplasmic reticulum (Fig. 1). Phospholipids which are the major components of biological membranes are transported to subcellular organella membranes. Physical properties of phospholipids are desided by their acyl residue compositions. Changes in the physical state of membrane phospholipids have been shown to affect several membrane functions (5, 6). Therefore it is necessary to study the effects of dietary fatty acids on phospholipid metabolism in animal. Animal cells in culture

appear to be a valuable tool for this purpose, since we can control the amount and variety of fatty acids by using refined growth medium.

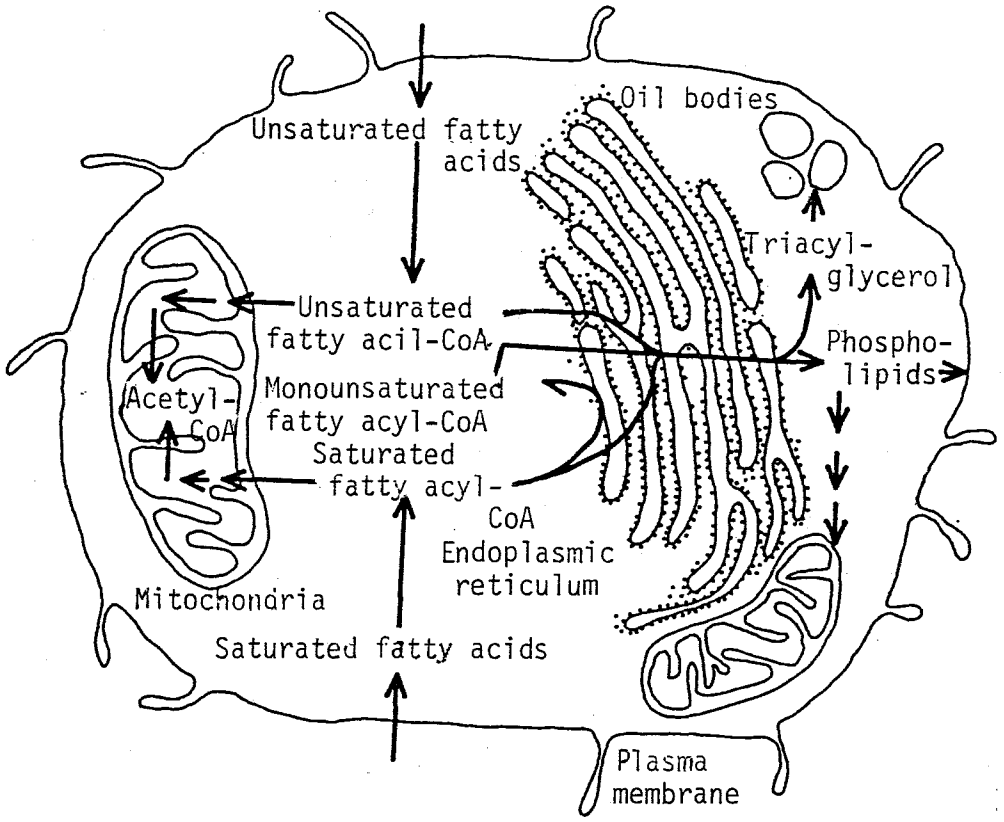

Fig. 1 METABOLISM OF FATTY ACID IN ANIMAL CELL

In this thesis, the toxic effects of dietary fatty acids on the animal cells, chinese hamster lung fibroblasts V79, are investigated from the standpoint of phospholipid metabolism. In chapter II, it is described that V79-R cells which can grow in lipid-free medium were isolated from V79 cells and that the difference in requirement for monoenoic fatty acid between both cell lines is due to the difference

in stimulation of stearoyl-CoA desaturase activity in lipid-starved culture. In chapter III, The occurrence of *cis*-vaccenic acid, which is a positional isomer of oleic acid, in phospholipids of V79-R cells is treated. In chapters IV, V and VI, the effects of palmitic, erucic and *trans*-monoenoic fatty acids on V79-R cells are described.

II MONOENOIC FATTY ACID REQUIREMENT IN V79 CELLS

II-1 INTRODUCTION

The finding of an Escherichia coli unsaturated fatty acid auxotroph (7) has contributed to dramatic development of studies on fluidity of biological membranes and membrane lipid metabolism in bacteria. Several animal cell mutants defective in the enzymes related to phospholipid (8,9) and sterol syntheses(10,11) have been isolated. Though a CHO mutant which required both cholesterol and monoenoic fatty acid simultaneously has been isolated (10), no unsaturated fatty acid auxotroph has been found yet.

V79 Fibroblasts were isolated from male chinese hamster lung by Ford and Yerganian (12) and have served as excellent experimental cells in the field of induction of mutation (13),cell adhesion (14,15), and lipid metabolism (10,11). We isolated V79-R from V79 cells as a cell line which could grow in lipid-starved culture. Growth of V79-R cells was inhibited by supplementation with palmitic (chapter IV) and erucic acids (chapter V) to the medium. These fatty acids inhibited the synthesis of phospholipids with two octadecenoic acids. This suggested that V79-R cells require phospholipid molecular species with two octadecenoic acids. Hence, it is postulated that failure in growth of V79 cells in the lipid-starved medium may depend on shortage of unsaturated fatty acids.

In animal cells, octadecenoic acids are formed from stearoyl- and palmitoyl-CoA by Δ9-desaturation and elongation (18). Stearoyl-CoA desaturase has been shown to be induced in lipid-starved animal cells (19). The present paper reports that V79 cells require monoenoic fatty acids for growth and that this requirement arises from the defect in stimulation of stearoyl-CoA desaturase activity in lipid-starved culture.

II-2 MATERIALS AND METHODS

Materials

[1-^{14}C]Acetyl-CoA(55 mCi/mmol), sodium [^{14}C] bicarbonate (57.1 mCi/mmol) and [1-^{14}C]stearoyl-CoA (55 mCi/mmol) were purchased from Amersham International Limited, Amersham. [1-^{14}C]Acetic acid (56 mCi/mmol) was from New England Nuclear Corp., Boston, MA. Fetal calf serum was from Microbiological Associates Bioproducts, Walkerville, MD. Thin-layer plates were purchased from Merck, Darmstadt. Unisil (100-200 mesh) was from Clarkson Chemical Company Inc., Williamsport, PA. All other chemicals were of analytical grade.

Cells and growth medium

Chinese hamster V79 lung fibroblasts were the gift of Drs. Tokindo Okada and Masatoshi Takeichi, Department of Biophysics, Faculty of Science, Kyoto University. V79-R Cells ,which can grow in lipid-free medium, were isolated from V79 cells . Both cell lines were routinely main-

tained in MEM (20) supplemented with 6% (v/v) fetal calf serum. Lipid-free medium was prepared from MEM supplemented with 6% (v/v) delipidated fetal calf serum. Delipidated serum was prepared according to the method described by Cham and Knowles (21). The cells were incubated at 37°C under a 5% CO_2 atmosphere at 100% relative humidity.

Fatty Acid Analysis

The cell monolayers were rinsed with 2 ml of 1 mM EDTA in Ca^{2+}-, Mg^{2+}-free Hank's solution (CMF) and harvested with 0.25% trypsin. The cells were collected and washed twice with 10 ml of 1 mM EDTA in CMF by centrifugation at 100 x g for 5 min. The cellular lipids were extracted by the method of Bligh and Dyer (22). Phospholipids were separated from other lipids by thin-layer chromatography with a solvent system of hexane/ diethylether/ acetic acid (50:50:1, by vol). Isolated phospholipids were transmethylated with sodium methoxide, and the fatty acid methyl esters were determined by gas liquid chromatography using a 10% Silar-10C column according to the method of Kito et al. (23).

Incorporation of [1-^{14}C]acetic acid into fatty acids and phospholipid molecular species in V79 and V79-R cells

Cultures were incubated with 1 μCi of [1-^{14}C]acetic acid (56 mCi/mmol) in 2 ml of lipid-free medium for 60 min at 37°C. To stop the uptake of radioactive acetic acid by the cells, 1 ml of 20 mM sodium acetate was added into the medium. The medium was then removed and the cultures were

washed with 3 x 2 ml of 1 mM EDTA in CMF. The cells were dispersed with trypsin, and cellular lipids were extracted (22). In the experiment of incorporation of [^{14}C]acetic acid into fatty acids, 100 µg of oleic and palmitic acids were added as carriers into the extracts. The extracts were evaporated to dryness and saponified for 30 min at 80°C after adding 0.5 ml of 20% KOH in methanol and 0.5 ml of distilled water. Fatty acids were then extracted with 3 x 2ml of diethylether after acidification of the alkaline extract with 7.5 M HCl. The fatty acids were methylated with boron trifluoride for 5 min at 100°C. The methylesters of saturated and monoenoic fatty acids were separated on $AgNO_3$-impregnated thin-layer plates with a solvent system of benzene/ chloroform/ methanol (196:4:0.2, by vol.). The fractions corresponding to saturated and monoenoic fatty acid esters were scraped off from the plates. The fatty acid esters were extracted from the silica gel once with 4 ml of diethylether overnight and twice with 2 ml of diethylether. Silver nitrate in the extracts was removed by the method of Åkesson (24). The radioactivities in the extracts were measured in a toluene-based scintillation fluid. In the experiments of incorporation of [^{14}C]acetic acid into phospholipid molecular species, 1 mg of phospholipids from V79-R cells grown in lipid-free medium were added as carrier into the lipid extracts from the cells which took up [^{14}C]acetic acid. The phospholipids were isolated by Unisil-column

chromatography and transformed into 1,2-diacyl-3-acetylglycerol (23). The separation of 1,2-diacyl-3-acetylglycerol molecular species was carried out by the method of Kito et al. (23). The fractions corresponding to molecular species with two monoenoic fatty acids, one monoenoic and one saturated fatty acids, and two saturated fatty acids were scraped off from the thin-layer plates. The extraction of 1,2-diacyl-3-acetylglycerol and removal of silver nitrate from the extracts were carried out as described above, and the radioactivities were measured in a toluene-based scintillation fluid.

Preparation of cell extract

Cells in monolayer were washed twice with 1 mM EDTA in CMF and removed by scraping with a rubber policeman. The cells were collected and washed twice with 1 mM EDTA in CMF by centrifugation at 100 x g for 5 min. All procedures mentioned below were carried out at 4°C. The cell pellet was resuspended in ice-cold buffer containing 50 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 5 mM 2-mercaptoethanol, and 1 mM EDTA, and homogenized in a Dounce homogenizer. The homogenate was centrifuged at 750 x g for 10 min. The supernatant was centrifuged at 10000 x g for 10 min to precipitate mitochondria. The supernatant at 10000 x g was fractionated by further centrifugation at 100000 x g for 60 min. The pellet at 100000 x g (microsomal fraction) was resuspended in 1 mM Tris-HCl, pH 7.5. The supernatant at

100000 x g was dialyzed twice against 100 vol. of 50 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 10 mM potassium citrate for assay of acetyl-CoA carboxylase, or 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM dithiothreitol and 1 mM EDTA for assay of fatty acid synthetase activity.

Enzyme assays

Acetyl-CoA carboxylase activity was assayed in the dialyzed cytosolic fraction mentioned above by the method of Nakanishi and Numa (25). The enzyme solution was preincubated at 37°C for 30 min in a mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM potassium citrate, 10 mM $MgCl_2$, 3 mM glutathione, and 0.6 mg bovine serum albumin per milliliter to attain its full activation. Fatty acid synthetase activity was assayed by the method of Hsu et al. (26). Microsomal stearoyl-CoA desaturase activity was assayed by the method of Jones et al. (27).

Protein content

Protein was determined by the method of Lowry et al. (28) using bovine serum albumin as the standard.

II-3 RESULTS

Oleic acid requirement of V79 cells

Fig. 1 shows the growth of V79 and V79-R cells in the medium with or without lipids. Both cell lines proliferated in the medium supplemented with 6% fetal calf serum. V79-R

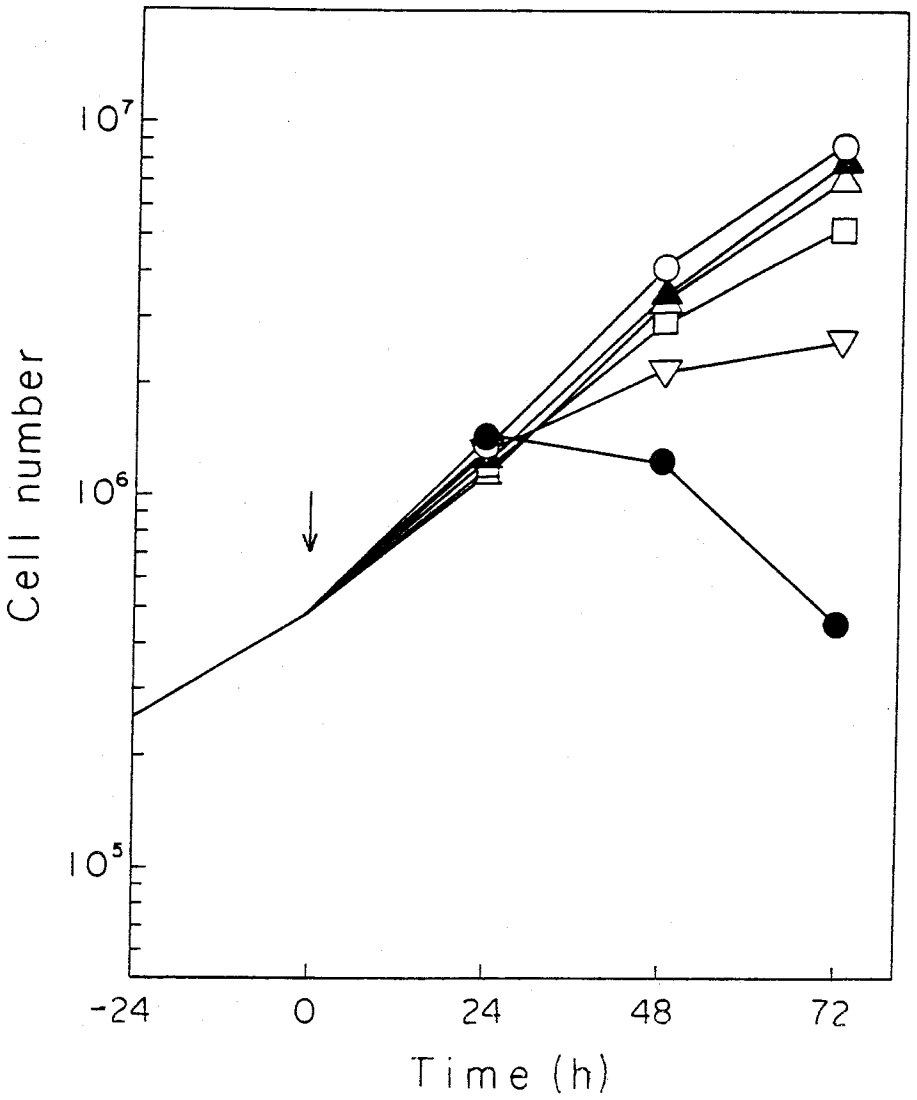

Fig. 1 Requirement for oleic acid by V79 cells. 2.5 x 10^5 cells were transferred to a culture dish containing 4 ml of MEM supplemented with 6% fetal calf serum (complete growth medium). After 1 day (arrow), cultures were incubated in 4 ml of lipid-free medium supplemented with various concentrations of oleic acid complexed to albumin (29) or left to grow in complete growth medium. Growth of V79 cells in complete growth medium, ○ ; in lipid-free medium without oleic acid, ● ; in lipid-free medium supplemented with 10 μM oleic acid, ▽ ; in lipid-free medium supplemented with 25 μM oleic acid, △ ; in lipid-free medium supplemented with 50 μM oleic acid, ▲ ; V79-R cells in lipid-free medium, □ .

Cells continued to grow after removal of lipids from the culture medium. However, removal of lipids from the culture medium caused the cessation of growth and cell lysis of V79 cells. The requirement for lipid(s) by V79 cells was satisfied by octadecenoic acids. V79 Cells proliferated in the presence of 25 μM or more oleic acid as well as in the medium supplemented with serum lipids. Cis-vaccenic acid, a positional isomer of oleic acid, was also effective (data not shown). Though palmitoleic, linoleic, linolenic, and arachidonic acid supported growth of V79 cells at a concentration of 50 μM, the cells divided more slowly than did they in the medium containing serum lipids (data not shown). The requirement for monoenoic fatty acids was seen against V79 cells of 0 to 20 generations after thawing of the frozen cells. Cells which were able to grow in the lipid-free medium were obtained during a long-term culture.

Fatty acid composition of phospholipids in V79 and V79-R cells

When V79 and V79-R cells were grown in the medium containing serum lipids, cellular phospholipids were composed of 11.1 and 9.7 % arachidonic acid, 3.8 and 4.4 % linoleic acid, 3.5 and 3.2 % docosapentaenoic acid, and 3.5 and 3.7 % docosahexaenoic acid, respectively. These polyenoic fatty acids in phospholipids were derived from exogenous lipid(s) in the serum. Starvation of the exogenous lipids by shift-down of the medium to lipid-free medium

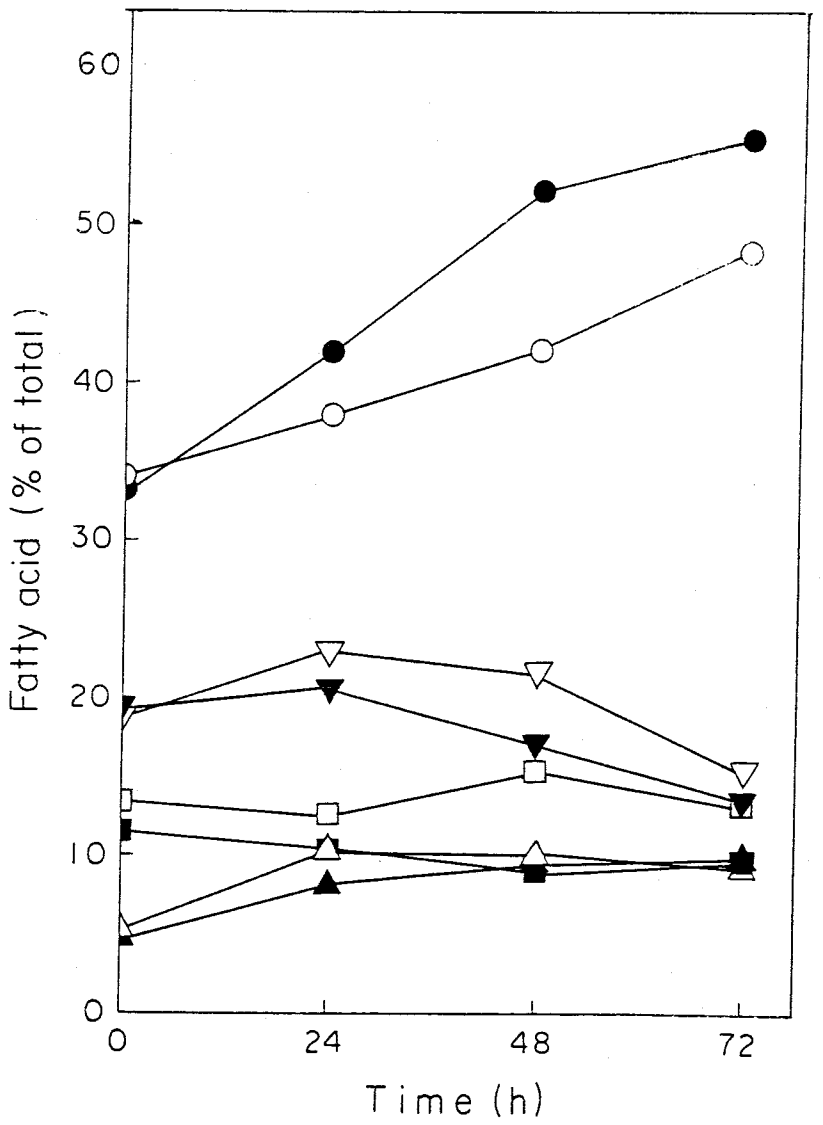

Fig. 2 Changes in fatty acid composition of phospholipids in V79 and V79-R cells after deprivation of lipid . Multiple 100-mm-diameter cultures were each seeded with 7 x 10^5 V79 (open symbols) and V79-R cells (filled symbols) in 10 ml of complete growth medium. After 1 day (at zero time), the medium was replaced with lipid-free medium as described in the legend of Fig. 1. Replacement of the medium was carried out every day. Phospholipids were isolated at the indicated times from cells, and the fatty acid composition was determined as described in "MATERIALS AND METHODS." Octadecenoic acid, ○ ● ; hexadecenoic acid, △ ▲ ; stearic acid, □ ■ ; palmitic acid, ▽ ▼ . Values represent the mean weight % of total fatty acid.

decreased polyenoic fatty acids and increased octadecenoic acid in V79-R cells (Fig. 2). The octadecenoic acid content increased more slowly in V79 cells than in V79-R cells. The percentages of stearic and palmitic acids in V79 cells were higher than those in V79-R cells after lipid deprivation. These results suggest that there is a difference in the rate of Δ9-desaturation reaction of saturated fatty acids between V79 and V79-R cells.

Syntheses of fatty acids and phospholipid molecular species in lipid-starved V79 and V79-R cells

The rate of de novo fatty acid synthesis was determined in the lipid-starved cultures by incubating the cells with [^{14}C]acetic acid (Fig. 3A). In V79-R cells, there were marked increases in incorporation of [^{14}C]acetic acid into monoenoic and saturated fatty acids after the removal of lipids from the medium, whereas the incorporation varied little during the first 48 h after the shift-down of the medium and then declined in V79 cells. Incorporation of [^{14}C]acetic acid into saturated and monoenoic fatty acids in V79 cells was far less than that in V79-R cells at 48 h after the shift-down of medium. The ratio of monoenoic fatty acids converted from the saturated fatty acids de novo synthesized from [^{14}C]acetic acid in the lipid-starved medium were shown in Fig. 3B. After the removal of lipids from culture medium, the ratio of monoenoic fatty acids in V79-R cells was increased to about twice that at zero time.

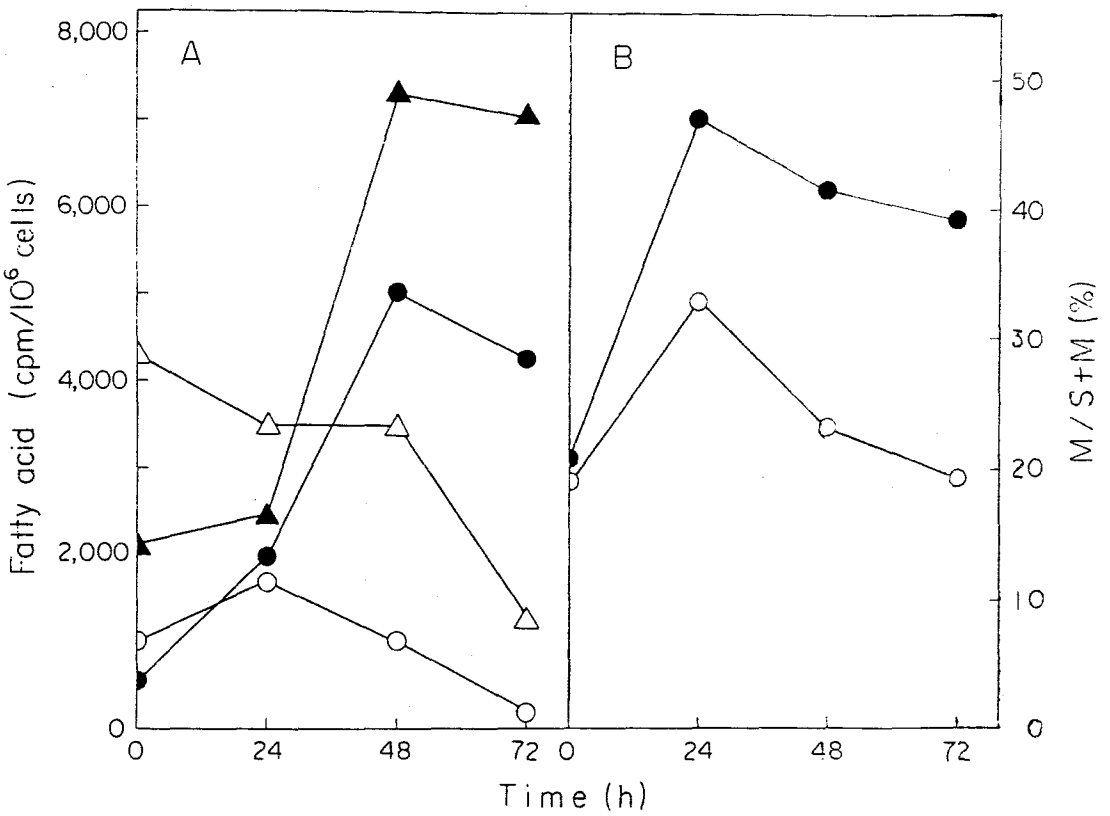

Fig. 3 Changes in cellular fatty acid synthesis after lipid deprivation. 2.5×10^5 cells were transferred to 60-mm-diameter dishes containing complete growth medium. After 1 day (at zero time), the cells were rinsed as described in the legend of Fig. 1 and incubated in lipid-free medium. The medium was replaced with lipid-free medium every day. A, Incorporation of [1- ^{14}C] acetic acid into fatty acids of V79 (open symbols) and V79-R cells (filled symbols) for 60 min at the indicated times was determined as described in "MATERIALS AND METHODS." Radioactivity in saturated fatty acids, △ ▲ ; radioactivity in monoenoic fatty acids, ○ ● . Values represent the mean of two experiments. B, Values were obtained from the results shown in A by dividing the radioactivity in monoenoic fatty acids by the sum of radioactivity in monoenoic and saturated fatty acids and multiplying by one hundred.V79 Cells, ○ ; V79-R cells, ● .

The ratio decreased to the same level as that at zero time through a transient increase in V79 cells. It was always lower in V79 cells than that in V79-R cells throughout

the incubation. Similar results were obtained when the radioactivity was shown per mg protein (data not shown). These results suggest that the de novo synthesis of fatty acids and desaturation of saturated fatty acids in V79 cells was scarcely induced after lipid deprivation.

When V79-R cells were grown in lipid-free medium, the phospholipid molecular species synthesized were mainly composed of molecular species with two saturated fatty acids, one saturated and one monoenoic fatty acids and two monoenoic fatty acids (17). The incorporation of [^{14}C]acetic acid into those phospholipid molecular species in lipid-free medium is shown in Fig. 4A. After 24-h incubation in lipid-free medium, the extent of incorporation of [^{14}C]acetic acid into the molecular species with two saturated fatty acids, one saturated and one monoenoic fatty acids, and two monoenoic fatty acids in V79-R cells were 7.7-, 13.8-, and 23-times those at zero time, respectively. However, the extent of incorporation in V79 cells was 3.6-, 4.1-, and 3.6-times that at zero time, respectively. After the removal of lipids, the level of incorporation of [^{14}C]acetic acid into the molecular species with two monoenoic fatty acids and one saturated and one monoenoic fatty acids in V79 cells was lower than that in V79-R cells. However, there was little difference between the V79 and V79-R cells in their incorporation of [^{14}C]acetic acid into the molecular species with two saturated fatty acids. The distribution of monoenoic

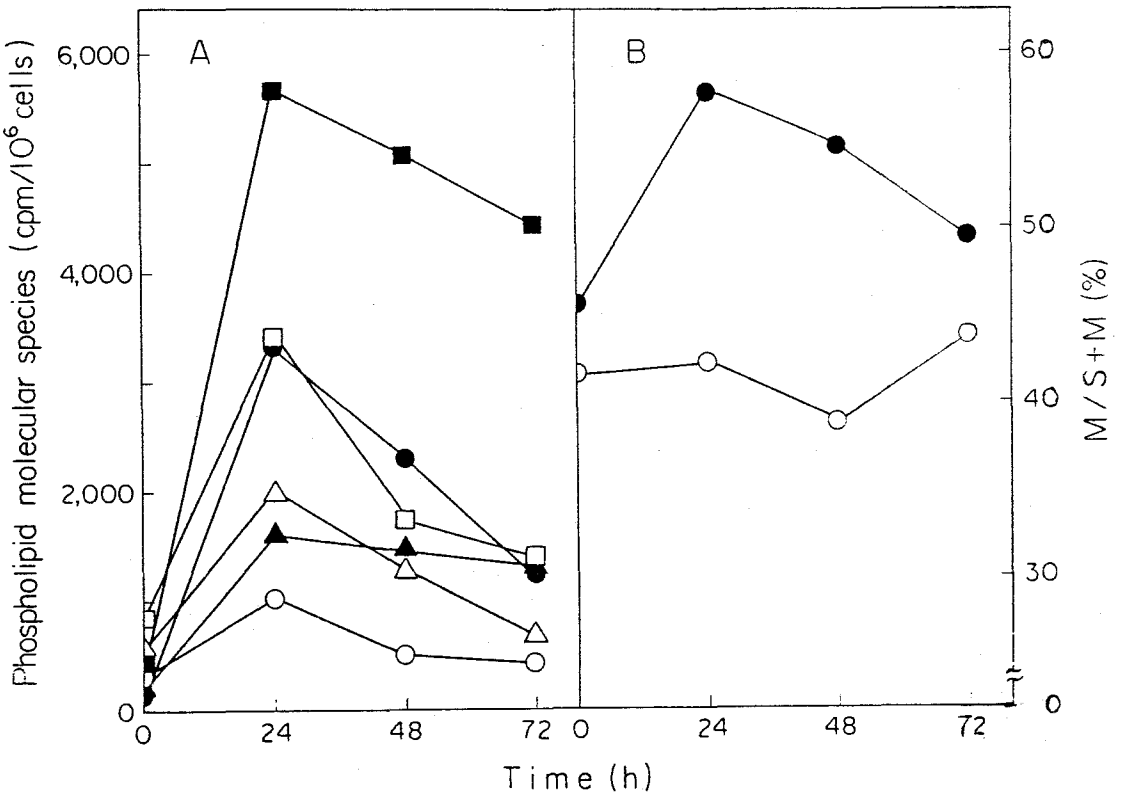

Fig. 4 Changes in cellular phospholipid molecular species synthesis after lipid deprivation. Cells were grown as described in the legend of Fig. 3. A, Incorporation of [1- ^{14}C]acetic acid into phospholipid molecular species of V79 (open symbols) and V79-R cells (filled symbols) for 60 min at the indicated times was determined as described in " MATERIALS AND METHODS." Radioactivity in phospholipids with two monoenoic fatty acids, ○ ● ; in phospholipids with one saturated and one monoenoic fatty acids, □ ■ ; in phospholipids with two saturated fatty acids, △ ▲ . Values represent the mean of two experiments. B, Values were obtained from the results in A by dividing the sum of radioactivity in phospholipids with two monoenoic fatty acids and a half radioactivity in phospholipids with one saturated and one monoenoic fatty acids by the sum of the radioactivity in the total phospholipid molecular species and multiplying by one hundred. V79 Cells, ○ ; V79-R cells, ● .

acids in the total radioactive fatty acids of phospholipids was kept constant in lipid-starved V79 cells, whereas it increased in V79-R cells under lipid deprivation (Fig. 4B).

Enzyme activities related to fatty acid synthesis

The differences between V79 and V79-R cells in the rate of incorporation of [^{14}C]acetic acid into fatty acids (Fig. 3) led us to determine the specific activities of acetyl-CoA carboxylase, fatty acid synthetase, and stearoyl-CoA desaturase (Table I), which have been shown to play an important role in regulation of fatty acid biosynthesis (30-33). There was no difference in the specific activities of acetyl-CoA carboxylase and fatty acid synthetase between the cell lines at zero time. V79 and V79-R cells showed similar stimulation profiles in the specific activities of acetyl-CoA carboxylase (1.6- and 1.7-fold) and fatty acid synthetase (1.5- and 1.5-fold) at 48 h after the lipid deprivation. An increase in the formation of monoenoic fatty acids in V79-R cells (Fig. 3B) was corroborated by 3-fold stimulation of activity of stearyl-CoA desaturase, which is a key enzyme for monoenoic fatty acid synthesis in animal cells (32,33). No stimulation of stearoyl-CoA desaturase activity was observed in V79 cells. Hence, the deficiency in the stimulation may explain the lower desaturation ability of V79 cells than that of V79-R cells after lipid deprivation (Fig. 3B).

TABLE I

ACTIVITIES OF FATTY ACID BIOSYNTHETIC ENZYMES IN V79 AND V79-R CELLS

Enzyme activities were determined as described in "MATERIALS AND METHODS". Values are given as mean ± S.E. for three to six determinations.

Enzyme	Cell	Specific activity	
		zero time	48 h after shift-down
		nmol/min/mg protein	
Acetyl-CoA carboxylase	V79	5.51 ± 0.05	8.07 ± 0.15
	V79-R	5.43 ± 0.18	9.32 ± 0.15
Fatty acid synthetase	V79	0.78 ± 0.03	1.28 ± 0.06
	V79-R	0.89 ± 0.07	1.34 ± 0.09
		pmol/min/mg protein	
Stearoyl-CoA desaturase	V79	58.4 ± 2.4	31.5 ± 2.7
	V79-R	108.5 ± 15.7	320.3 ± 3.1

II-4 DISCUSSION

In this chapter, I have demonstrated that V79 cells required monoenoic fatty acids for growth and that this requirement is caused by deficiency in the cellular monoenoic fatty acid formation. The reduced formation of monoenoic fatty acids in V79 cells may be due to a defect in the stimulation in stearoyl-CoA desaturase activity after removal of lipids from the growth medium.

V79 Cells could not grow in the medium supplemented with delipidated serum. The lipids in fetal calf serum contained octadecenoic, linoleic, and arachidonic acids as major unsaturated fatty acids (data not shown). Oleic and cis-vaccenic acid completely satisfied the requirement for

serum lipid(s) by V79 cells. We reported that phospholipids of V79-R cells are composed of molecular species with two saturated fatty acids, one saturated and one monoenoic fatty acids and two monoenoic fatty acids (mainly, two octadecenoic acids) when the cells were grown in lipid-free medium (chapter V). The addition of palmitic and erucic acids to lipid-free medium resulted in the serious inhibition of growth of V79-R cells and inhibition of biosynthesis of phospholipid molecular species with two monoenoic fatty acids (chapter IV, V). These results strongly suggest that formation of the molecular species with two monoenoic fatty acids is essential for growth of V79-R cells when exogenous lipids are not supplied. I have shown here that in lipid-deprived V79 cells the formation of phospholipid molecular species containing monoenoic fatty acids (mainly, molecular species with two monoenoic fatty acids) was lower than those in lipid-starved V79-R cells (Fig.4). This may be due to the insufficient formation of the molecular species with monoenoic fatty acids. The results obtained with [^{14}C] acetic acid are consistent with the observation that the distribution of octadecenoic acid in phospholipids of V79 cells was lower than that in V79-R cells after lipid deprivation (Fig. 2).

In lipid-starved V79 cells, there was no stimulation of de novo synthesis of fatty acids from[^{14}C]acetic acid as was observed in V79-R cells, and the desaturation rate of

de novo synthesized saturated fatty acids to monoenoic fatty acids was lower than that in V79-R cells. The lower formation of molecular species with two monoenoic fatty acids and one saturated and one monoenoic fatty acids in V79 cells than in V79-R cells may be due to insufficient synthesis of monoenoic fatty acids. This was caused by the low specific activity of the microsomal stearoyl-CoA desaturase. V79 Cells had the specific activity of stearoyl-CoA desaturase which is at a level similar to that reported in other animal cells by other workers (34), and was about one half of that in V79-R cells at zero time. Thus, this cell line was not defective in stearoyl-CoA desaturase. Stearoyl-CoA desaturase activity of animal cells is regulated under various hormonal and dietary conditions (34-36). The stimulation of stearoyl-CoA desaturase activity in V79-R cells by lipid deprivation is consistent with the results of other workers that stearoyl-CoA desaturase in human skin fibroblasts is enhanced by growth in lipid-free medium and inhibited by supplementation with cis-unsaturated fatty acids like as linoleic, oleic, and cis-vaccenic acids (37). In contrast to V79-R cells, V79 cells were shown unable to stimulate stearoyl-CoA desaturase activity after lipid deprivation. Total fatty acid synthesis in V79 cells after removal of lipids was lower than that in V79-R cells. This cannot be accounted for by the enzyme levels, since acetyl-CoA carboxylase and fatty acid synthetase activities in V79 cells were

increased in a similar manner in V79-R cells. There is a possibility that the accumulation of saturated fatty acids owing to the weak desaturase activity in V79 cells may cause the inhibition of acetyl-CoA carboxylase activity. Palmitoyl-CoA has been shown to be an allosteric inhibitor of this enzyme (38).

Finally, in this chapter, I report that V79 cells are defective in stimulation of stearoyl-CoA desaturase activity.

III DISTRIBUTION OF cis-VACCENIC ACID IN CHINESE HAMSTER V79-R CELLS

III-1 INTRODUCTION

Many studies have reported the distribution of positional isomers of octadecenoic acid in bacteria (39), plants (40,41) and animals (42-45). Octadecenoic acids in lipids are usually composed of Δ9- and Δ11-isomers (oleic and cis-vaccenic acids) in animal cells. It is difficult to examine a sole endogenous lipid metabolism in an animals, since it is affected by dietary exogenous lipids. Hence, to determine distribution of cis-vaccenic acid in lipids in animal cells, the effects of exogenous lipids on the distribution have to be excluded. V79-R Cells, which can grow in lipid-free medium (chapter II), may be a valuable tool for this purpose.

In this chapter, I describe the relationship between lipid metabolism and cis-vaccenic acid in V79-R cells.

III-2 MATERIALS AND METHODS

Materials

[1-^{14}C]Palmitoyl-CoA (52mCi/mmol) and [1-^{14}C]stearoyl-CoA (55mCi/mmol) were purchased from Amersham International plc., Amersham, Buckinghamshire. Thin-layer plates (No. 5724) were purchased from Merck, Darmstadt. All other chemicals were of analytical grade.

Cells

Chinese hamster V79-R lung fibroblasts (chapter II) were used throughout this study. Cells were routinely maintained in Eagle's minimum essential medium supplemented with 6% delipidated fetal calf serum. Delipidated serum was prepared according to the method described by Cham and Knowles (21). The cells were incubated at 37°C under a 5% CO_2 atmosphere at 100% relative humidity.

Preparation of mitochondrial and microsomal fractions

The cell monolayers were rinsed twice with 2 ml of Ca^{2+}-, Mg^{2+}-free Hank's solution containing 1 mM EDTA and cells were scraped off with a rubber policeman. Cells were collected and washed with ice-cold 10 mM Tris-HCl buffer, pH 7.5 containing 0.25 M sucrose (buffer A) by centrifugation at 100 x g for 5 min. The cell pellets were resuspended in buffer A (5 x 10^7 cells /ml) and homogenized in a Dounce homogenizer. The homogenates were centrifuged at 750 x g for 10 min. The supernatants from the centrifugation were centrifuged at 10000 x g for 5 min. The pellets were washed twice with buffer A by centrifugation at 10000 x g for 5 min. The pellets were resuspended with buffer A (mitochondria fraction). The preparation of microsomes for fatty acid analysis was obtained from the supernatant at 10000 x g by the sucrose density gradient centrifugation described by Green et al. (46). Microsomes for assay of acyl-CoA desaturase activity were prepared from the supernatant at

10000 x g by centrifugation at 100000 x g for 60 min. The pellet at 100000 x g was resuspended in 1 mM Tris-HCl buffer, pH 7.5 (microsomal fraction).

Lipid analysis

Lipids were extracted by the method of Bligh and Dyer (22). Extracted lipids were separated by thin-layer chromatography as described in chapter IV. For fatty acid analysis, lipids were transmethylated with sodium methoxide, and the fatty acid methyl esters were determined by isothermal analysis using a Shimadzu 7A gas liquid chromatograph equipped with a 50 m x 0.25 mm flexible glass capillary column coated with SS-10 at 200°C. Positional isomers of monoenoic fatty acid methyl esters were separated by this gas liquid chromatography as shown in Fig. 1.

Enzyme assays

Fatty acid desaturase [EC 1.14.99.5] activity was measured by the method of Jones et al. (27).

Protein Content

Protein was determined by the method of Lowry et al. (28) using bovine serum albumin as the standard.

III-3 RESULTS AND DISCUSSION

The fatty acid compositions of phosphatidylcholine (PC), phosphatidylethanolamine (PE), cardiolipin (CL), and triacylglycerol (TG) in cells grown in the medium supplemented with or without fatty acids were shown in Fig. 2. These

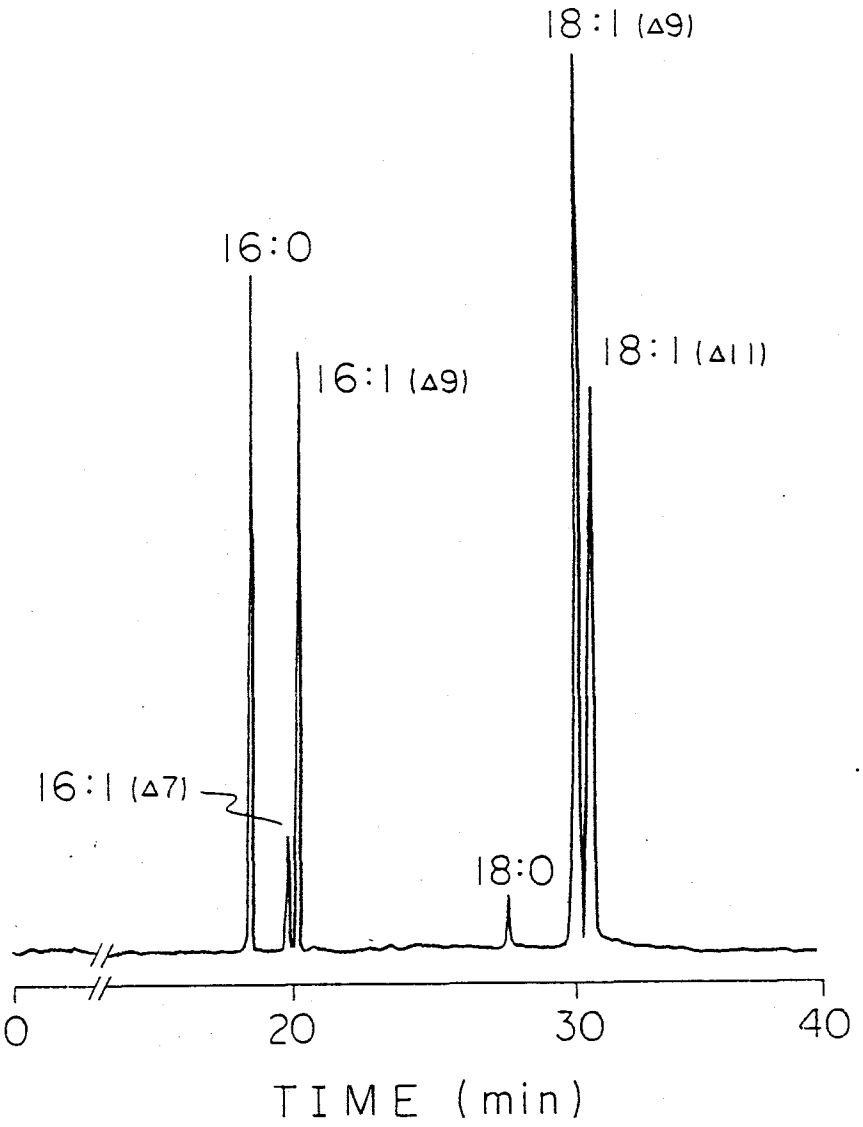

Fig. 1 Gas liquid chromatogram of fatty acid methyl esters. Conditions were described in MATERIALS AND METHODS.

lipids contained palmitic (16:0), hexadecenoic (16:1), stearic (18:0), and octadecenoic acid (18:1) as fatty acyl chains. When cells were grown in the medium supplemented with 6% delipidated serum, the major fatty acid in the lipids was 18:1 (about 60 to 70 %). In addition, 18:1 was composed of two positional isomers, oleic (18:1(Δ 9)) and

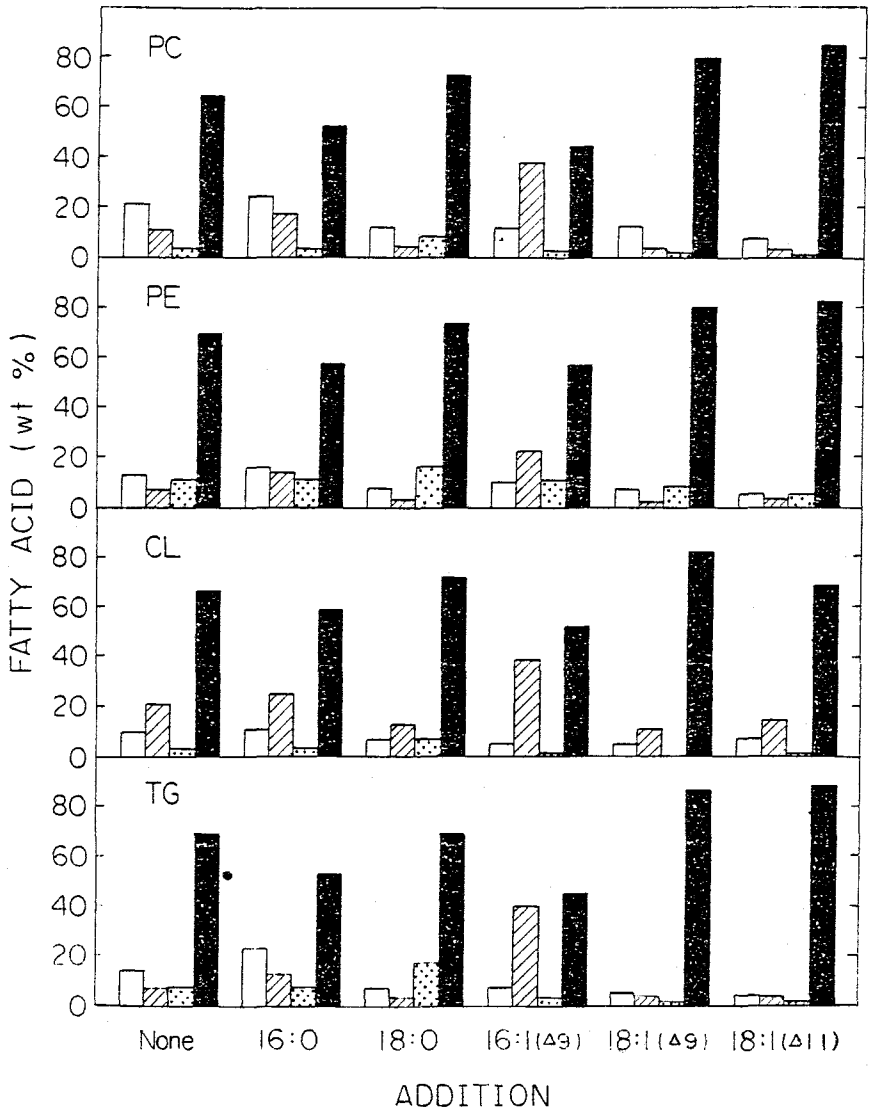

Fig. 2 Fatty acid compositions of lipids in V79-R cells supplemented with or without fatty acids. Multiple 100-mm-diameter cultures were each seeded with 7 x 10^5 cells in 10 ml of medium supplemented with 6% delipidated fetal calf serum. After 24 h, fatty acids (50 μM 16:0, 50 μM 18:0, 100 μM 16:1(Δ9), 100 μM 18:1(Δ9), 100 μM 18:1(Δ11)) complexed to bovine serum albumin (29) were added to the medium. After 24 h of incubation with fatty acids, lipids were extracted from the cells and the fatty acid composition was determined as described in MATERIALS AND METHODS. 16:0, □ ; 16:1, ▨ ; 18:0, ▩ ; 18:1, ■ . Values represent the mean weight percentage of total fatty acids with two experiments.

cis-vaccenic (18:1(△ 11)) acids (Fig. 3). 18:1(△ 9) is derived via △9-desaturation of stearoyl-CoA, whereas 18:1 (△ 11) is formed by △9-desaturation of palmitoyl-CoA and subsequent C_2-elongation in the animal (47). There was a difference in the distribution of 18:1(△ 9) and 18:1(△11) in the 18:1 fraction between the individual lipids from control cells. The distributions of 18:1(△11) in the 18:1 fraction of CL, TG, PC, and PE from control cells were 38, 24, 23, and 16%, respectively. More than 80% of the 16:1 fraction in the lipids was palmitoleic acid (16:1(△ 9)), which is a precursor of 18:1(△ 11). CL contained a rather higher content of both 18:1(△ 11) and 16:1 (20.5%) than did other lipids (about 6 to 9%).

Fatty acid compositions of cellular lipids were modified by the addition of 18:1(△ 9), 18:1(△11), 18:0, 16:0, and 16:1(△ 9) into the medium (Figs.2 and 3). Addition of 18:1(△ 9) and 18:1(△11) caused increases in the proportion of 18:1(△ 9) and 18:1(△11) in all lipids. Longer-chain fatty acids than 18:1 was not found in any lipids. We observed that the lipids in V79-R cells could incorporate exogenous long-chain monoenoic fatty acids such as erucic acid (chapter V). These results suggest that the elongation of monoenoic fatty acid beyond the 18 carbon chain did not occur in V79-R cells. Addition of 16:0 caused increases in the proportion of 16:0 and 16:1 with a concomitant decrease of 18:1. The decrease in the 18:1 fraction was due to the

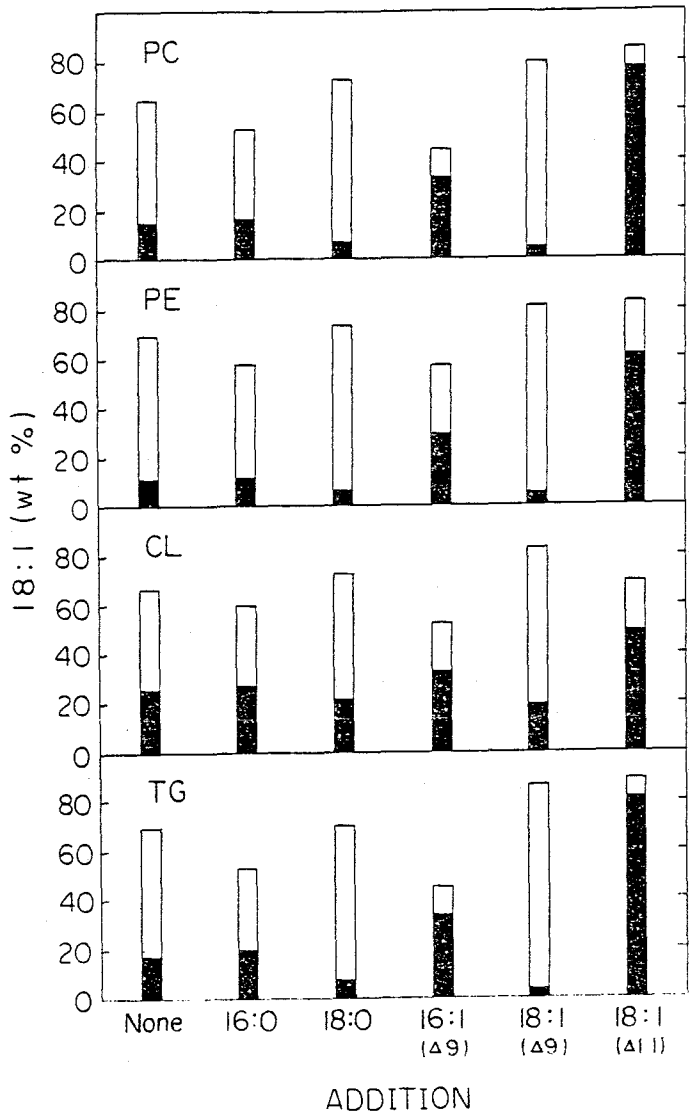

Fig. 3 Distribution of oleic and cis-vaccenic acids in the octadecenoic acid fraction in cellular lipids. Conditions were described in the legend of Fig. 2. 18:1(△ 9), □; 18:1(△ 11), ■. Values represent the mean weight percentage of total fatty acids with two experiments.

decrease of 18:1(△ 9). For example, the 18:1 fraction in PC decreased from 64.1 to 52.9% after 24 h of incubation in the medium containing 50 μM 16:0. Among 18:1 isomers, 18:1(△ 9) decreased from 49.5 to 37% whereas 18:1(△ 11) slightly increased from 14.6 to 15.9%. These results suggest that the

raised level of 16:0 in the cell caused by the addition of 16:0 into the medium resulted in the increase in the formation of 16:1(Δ 9) and 18:1(Δ 11). The desaturation rate from 16:0 to 16:1(Δ 9) may be faster than the elongation rate from 16:0 to 18:0 in the presence of a large quantity of 16:0 in V79-R cells. The addition of 16:1(Δ 9)into the medium caused a significant increase in 16:1(Δ 9) and 18:1 (Δ11) with the decrease in 16:0 and 18:1(Δ9). The increase in 18:1(Δ11) in cellular lipids may be explained by direct conversion of 16:1(Δ9) to 18:1(Δ11) by chain elongation. The addition of 18:0 caused the increases in the proportion of 18:1 fraction which were due to the increase in 18:1 (Δ 9). This suggests that 18:0 was desaturated rapidly to 18:1(Δ 9) in V79-R cells. There were the differences in the extent of modification by exogenous fatty acids between the individual lipids. This may be due to the difference in the turnover rates between the respective lipids.

We reported that the major phospholipid molecular species consisted of the species with two 18:1, and that the formation of this molecular species was essential for growth when V79-R cells were incubated in lipid-free medium (chapter IV, V). Although the distribution of 18:1(Δ 9) and 18:1 (Δ 11) in the 18:1 fraction was altered by the addition of 18:1(Δ 9) and 18:1(Δ11) to the medium, cell growth was not affected (data not shown). Thus, 18:1(Δ 9) and 18:1(Δ 11) may be substituted for each other in the cells' requirement.

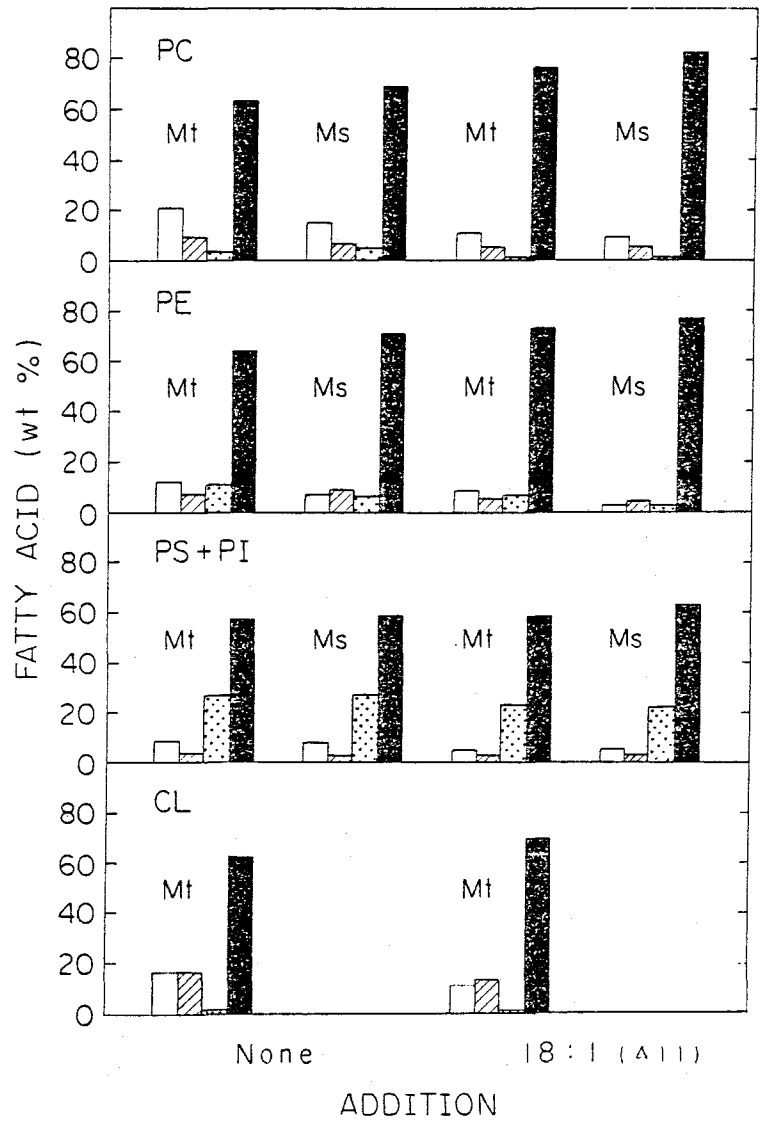

Fig. 4 Fatty acid composition of lipids in mitochondria and microsomes. 2 x 10^6 Cells were transferred to 100-mm-diameter dishes containing 10 ml of medium supplemented with 6% delipidated serum. After 24 h, 100 μM 18:1(Δ11) complexed to bovine serum albumin were added to the medium. After 24 h of incubation with 18:1(Δ11), mitochondria and microsomes were prepared from the cells and fatty acid composition of lipids was determined as described in MATERIALS AND METHODS. Mt, Mitochondria; Ms, microsome. Symbols were shown in the legend of Fig. 2. Values represent the mean weight percentage of total fatty acids with two experiments.

The fatty acid compositions of phospholipids in mitochondrial and microsomal fractions are shown in Figs.4 and 5. There was little difference in fatty acid compositions of PC, PE, and phosphatidylserine plus phosphatidylinositol(PS+PI) between mitochondria and microsomes in the control cells. The fatty acid compositions of phospholipids in mitochondrial and microsomal fractions were modified

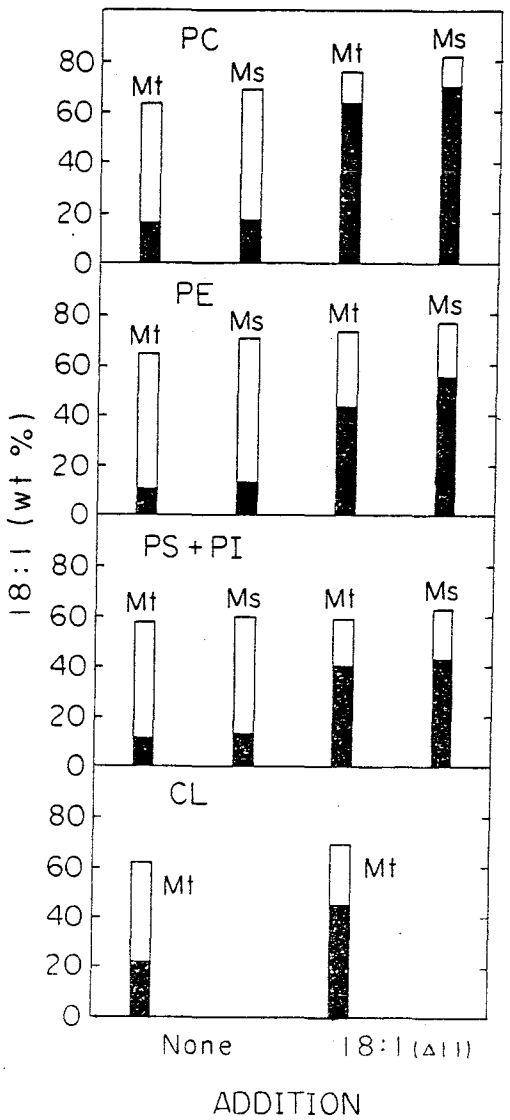

Fig. 5 Distribution of oleic and cis-vaccenic acids in the octadecenoic acid fraction in lipids from mitochondria and microsomes. Conditions were described in the legend of Fig. 4. 18:1(△ 9), □ ; 18:1(△11), ■ . Abbreviations were shown in the legend of Fig. 4. Values represent the mean weight percentage of total fatty acids with two experiments.

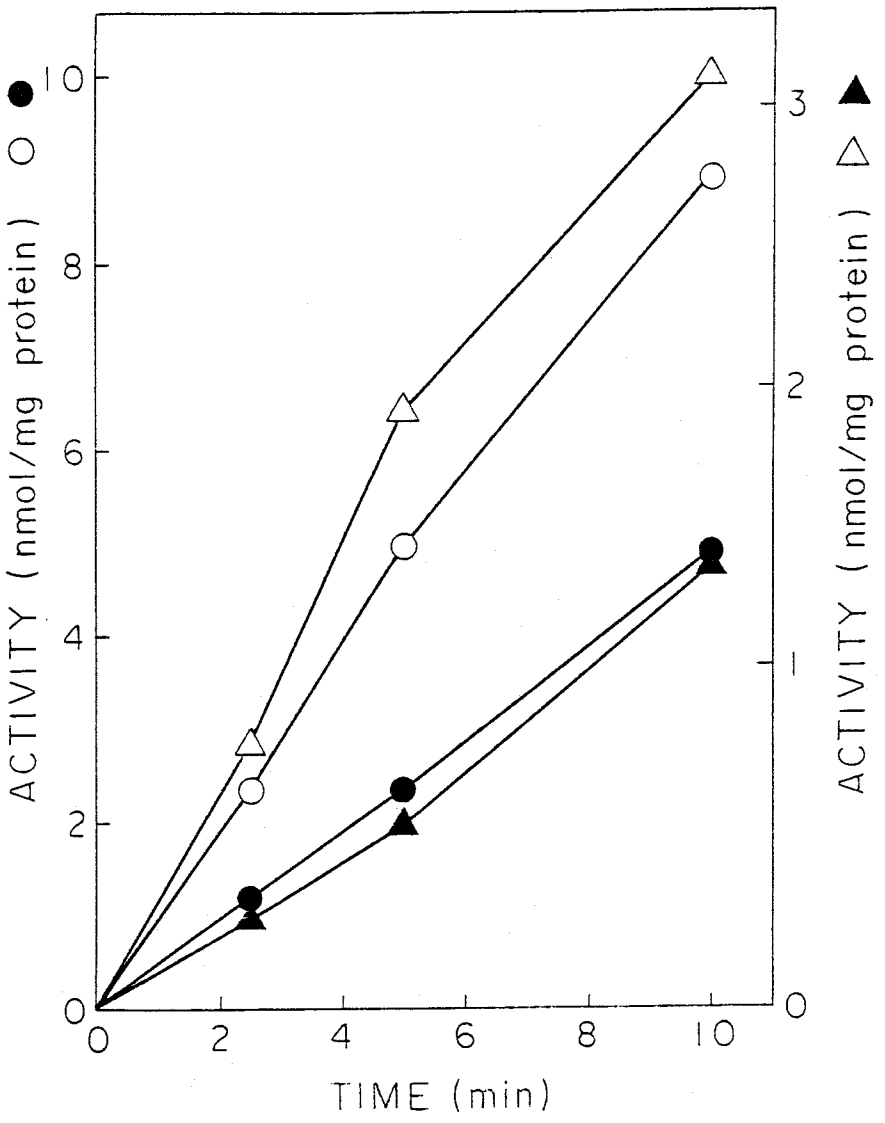

Fig. 6 Fatty acyl desaturase activity in mitochondrial and microsomal fractions from V79-R cells. Cells were grown in medium supplemented with 6% delipidated fetal calf serum. Preparations of mitochondria and microsome and enzyme assay were done as described in MATERIALS AND NETHODS. Fatty acyl desaturase reactions were done using stearoyl-(open symbols) and palmitoyl-CoA (filled symbols) as substrates for the indicated period. Mitochondrial fractions, △ ▲ ; microsomal fractions, ○ ● .

similarly by the addition of 18:1(Δ11) to the medium. The results strongly suggest that phospholipids synthesized in endoplasmic reticulum are transported rapidly to mitochon-

dria. CL is localized in the mitochondria. CL was a unique phospholipid which contained more 16:1(Δ 9) and 18:1(Δ 11) than did other phospholipids. Since the enzymes which synthesize CL are in the mitochondria (48, 49), there is a possibility that 16:1(Δ 9) and 18:1(Δ 11) are preferentially supplied to mitochondria. The possibility of a palmitoyl-CoA specific desaturase in mitochondria was examined by the measurement of fatty acyl desaturase activity in the mitochondrial fraction. The activity in the mitochondrial fraction was about one fourth of that in the microsomal fraction. There was no difference in substrate specificity (Fig. 6) and inhibition by KCN (data not shown) of fatty acyl desaturase between mitochondrial and microsomal fractions. The low fatty acyl desaturase activity in the mitochondrial fraction may be due to contamination by microsomes. Hence, it seems likely that the higher specificity of CL synthesizing enzyme system to the molecular species containing 16:1(Δ 9) and 18:1(Δ 11) may be a cause for high contents of 16:1(Δ 9) and 18:1(Δ 11) in CL.

IV PERTURBATION OF LIPID METABOLISM BY PALMITIC ACID IN CHINESE HAMSTER V79-R CELLS

IV-1 INTRODUCTION

The fatty acid composition of phospholipid of animal cells in culture was manipulated by supplementing the medium with fatty acids bound to bovine serum albumin (50-55). Triacylglycerol accumulated in cells upon the addition of fatty acids (54,56). However, cell growth is inhibited when saturated fatty acids are added to a medium containing delipidated serum (53, 55-58). Inhibition of cell growth does not occur in the presence of unsaturated fatty acids. The mechanism of toxicity of saturated fatty acids remains unclear.

This chapter describes the effect of palmitic acid on the growth, lipid metabolism and morphological characteristics of V79-R cells.

IV-2 MATERIALS AND METHODS

Chemicals

[1-^{14}C]Palmitic acid (53.82 mCi/mmol), [1-^{14}C] oleic acid (58.0 mCi/mmol), [2-^{3}H]glycerol (10.0 Ci/mmol), and Aquasol-2 were obtained from New England Nuclear Corp., Boston, MA. [U-^{14}C]Leucine (351.0 mCi/mmol) was from the radiochemical Centre Amersham, Amersham. Fetal bovine serum was purchased from Microbiological Associates Bioproducts, Walkerville,

MD. Trypsin (1:250) was from DIFCO Laboratories Detroit, MI. Fatty acid-free bovine serum albumin was the product of Miles Laboratories Inc., Elkhart, IN. Phospholipase C (Bacillus cereus) was obtained from Boehringer Mannheim, Mannheim. Thin-layer plates (No. 5721 and 5724) were from Merck, Darmstsdt. Unisil (100-200 mesh) was from Clarkson Chemical Company Inc., Williamstport, PA. All other chemicals were of analytical grade.

Cells and medium

Chinese hamster V79 lung fibroblasts were the gift of Dr. Tokindo Okada, Department of Biophysics, Faculty of Science, Kyoto University. Isolated V79 cells were adapted to grow in Eagle's minimum essential medium containing delipidated fetal bovine serum and redesignated "V79-R cells". Delipidated fetal bovine serum was prepared by the method of Cham and Knowles (21). The cells were grown in 4 ml of Eagle's minimum essential medium containing 6% delipidated serum (DMEM) at 37°C in a himidified atmosphere of 95% air/ 5% CO_2 in 60 mm Falcon tissue culture dishes. V79-R Cells were used throughout this study.

Lipid analysis

Lipids were extracted from cells by the methods of Blight and Dyer (22). Phospholipids and neutral lipids were separated from total lipid extracts by silicic acid chromatography using Unisil. Phospholipids were separated by thin-layer chromatography. The plates were first devel-

oped with the solvent system chloroform/acetone/methanol/water/acetic acid (100:100:50:10:4, by vol.) and then with chloroform/methanol/acetic acid/water (180:150:30:10, by vol) in the same direction. Neutral lipids were separated by thin-layer chromatography using the solvent system chloroform/benzene/methanol (25.5:500:15, by vol.). Lipids were identified by co-chromatography with authentic lipids. Phospholipid phosphate was determined according to the method of Bartlett (59). Portions of the extracts were used for determination of triacylglycerols and free fatty acids by the method of Biggs et al. (60) and of Novák (61), respectively. Sterols were determined by gas-liquid chromatography using a 2% OV17 column at 240°C. Phosphatidylcholine, phosphatidylethanolamine and triacylglycerol were separated on thin-layer chromatographic plates and transmethylated with sodium methoxide, and the fatty acid methyl esters were determined by gas-liquid chromatography using a 10% Silar 10-C column according to the method of Kito et al. (23). Phospholipid molecular species were determined according to the method of Kito et al. (23). For the analysis of triacylglycerol molecular species, triacylglycerols were separated by thin-layer chromatography as mentioned above, and the appropriate sections were scraped off and extracted with chloroform/methanol (2:1, by vol.). Molecular species of triacylglycerols were separated by silver nitrate thin-layer chromatography using the solvent system

chloroform/methanol (99:1, by vol.) (62).

Pulse-chase experiments

Cultures were initiated by seeding petri dishes with 1 x 10^5 cells. The culture medium was removed from the dish after incubation for 3 days and replaced with 4 ml of fresh DMEM containing albumin-bound [1-^{14}C]palmitic acid or [1-^{14}C]oleic acid (1 μCi). After 4-h incubation with radioactive fatty acids, the cells were washed with 2 ml of 0.5% fatty acid-free bovine serum albumin in Ca^{2+}- and Mg^{2+}-free Hank's solution (CMF). The medium was replaced with 4 ml of nonradioactive medium, and then the incubation was continued at 37°C for 24 or 48 h. The cells were dispersed with trypsin, and the lipids were extracted from the cell suspension. Lipids were separated by thin-layer chromatography. The radioactivities of lipid fractions were measured with a Packard liquid scintillation spectrometer (model 3385) in a solution composed of 0.6% PPO (w/v) and 0.05% POPOP (w/v) in toluene.

Phospholipid synthesis and membrane flow of phospholipids

Cultures were initiated by seeding dishes with 2.5 x 10^5 cells. After 18-h preculture, the cultures were washed with CMF and incubated with 4 ml of leucine-free DMEM and [U-^{14}C]leucine (1 μCi). After 6-h incubation, cold leucine (224 μg/ml) and palmitic acid (100 μM) were added to the medium, and the cells were cultured for a further 7 h. Then, [2-^{3}H]glycerol (10 μCi/dish) was added to the culture,

and the cultivation was continued for 17 h. Control cells were grown in the absence of palmitic acid. After the labeling, the culture dish was washed twice with 2 ml of 0.15 M NaCl, and the labeled cells were scraped off with a rubber policeman. Unlabeled carrier cells (7.5×10^6) were added to the labeled cells obtained from 10 dishes. These cells were collected by centrifugation at 100 x g for 5 min and washed twice with ice-cold buffer containing 0.25 M sucrose and 1 mM Tris-HCl, pH 7.2, and homogenized in a Dounce homogenizer (Weaton glass type B pestle) x 20 strokes. Microsome and plasma membrane fractions were prepared by the method of Baldassare et al. (63). Plasma membrane fractions contained negligible amounts of NADPH-cytochrome c reductase and succinate-dependent cytochrome c reductase. The radioactivities of ^3H and ^{14}C in the membrane fractions were counted in Aquasol.

Enzyme assays

Ouabain-sensitive (Na^+, K^+)-ATPase, NADPH-cytochrome c reductase and succinate-dependent cytochrome c reductase activities were assayed as markers of plasma membrane, microsome and mitochondria, respectively. Ouabain-sensitive (Na^+, K^+)-ATPase was assayed as described by Schimmel et al. (64). NADPH-dependent and succinate-dependent cytochrome c reductase activities were assayed spectrophotometrically according to the method of Sottocasa et al. (65).

Electron Microscopy

Cells were scraped from the dishes with a rubber policeman, centrifuged at 100 x g for 5 min, and the supernatant was discarded. Cold 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, was gently layered over the cell pellet. After 20 min at 4°C, the cell buttons were removed on paraffin wax, sliced into 1 mm^3 blocks, which were then washed in 0.5 M sucrose in 0.1 M phosphate buffer overnight in the cold and postfixed in 1% osmium tetroxide in 0.5 M sucrose and 0.5 M phosphate buffer. Dehydration with alcohol and propylene oxide was followed by embedding in Epon. Sections were cut on an Ultracut (American Optical Corporation, Reiohery) and stained with uranyl acetate and lead citrate prior to examination in a H-700H transmission electron microscope (Hitachi Co., Ltd., Japan).

Determination of cell size

After the cells were treated with trypsin, the diameters of 100 cells were measured with help of a micrometer eyepiece (Kyoto Rikakikai, Inc., Kyoto).

Measurement of cell volume

Cell volume was determined by the method of Tolbert et al. (66).

IV-3 RESULTS

Effect of saturated fatty acids on growth of V79-R cells

The effect of saturated fatty acids bound to bovine serum albumin on the growth of V79-R cells was examined

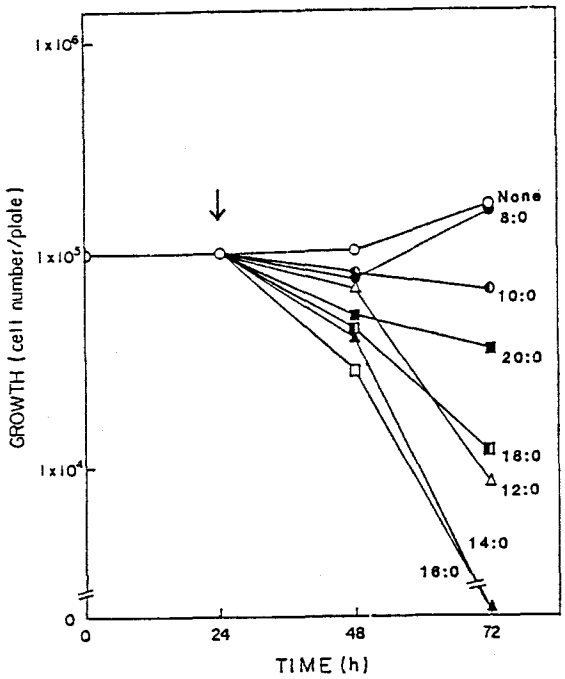

Fig. 1. The effect of saturated fatty acid supplementation on growth of V79-R cells. Cells (1×10^5) were transferred to a 60 mm tissue-culture dish in DMEM and then grown in the presence of the following fatty acids bound to fatty acid-free bovine serum albumin , which were added at the point indicated by an arrow. Final concentration of fatty acids were 100 μM: caprylic acid, 8 : 0; capric acid, 10 : 0; lauric acid, 12 : 0; myristic acid, 14 : 0, palmitic acid, 16 : 0; stearic acid, 18 : 0; arachidic acid, 20 : 0. Cells were dispersed with 0.25% trypsin in CMF. The number of cells was determined with a hemocytometer. Values represent the mean of two dishes.

(Fig. 1). Cell growth was not affected by the addition of caprylic acid at a concentration of 100 μM. Saturated fatty acids with longer chain lengths (C_{10}-C_{20}) caused a reduction in cell number. The addition of palmitic acid or myristic acid resulted in severe inhibition of cell growth. The degree of inhibition by palmitic acid was concentration dependent (Table I). At 75 and 100 μM, palmitic acid caused more than 90% inhibition of cell growth when

TABLE I. Inhibition of cell growth by palmitic acid and protective effect of oleic acid on the inhibition. Conditions except the concentration of fatty acids were described in Fig. 1. Values represent the mean of two dishes.

Fatty acid	Concentration (μM)	Cell growth (cells $\times 10^{-5}$/dish)
None		1.88
Palmitic acid	10	1.53
	25	1.35
	50	0.44
	75	0.14
	100	0.11
Palmitic acid (100 μM)+oleic acid	10	0.94
	25	1.28
	50	1.34

cultures were initiated by seeding dishes with 1 x 10^5 cells. However, in the presence of 100 μM palmitic acid, the number of cells was not reduced when cells were seeded at 2.5 x 10^5/dish (data not shown). Thus, greater the cell density, the less the inhibition at a fixed concentration of palmitic acid. Simultaneous addition of oleic acid protected cells from the inhibition by palmitic acid.

Effect of palmitic acid on cellular lipid composition

Total phospholipid per 10^6 cells was increased to twice that of control cells in the presence of palmitic acid (Table II). However, in the presence of oleic acid, phospholipid content decreased slightly. The addition of either palmitic acid or oleic acid resulted in a marked accumulation of cellular triacylglycerol. After 24-h incubation in the presence of palmitic acid, the accumulation of triacylglycerol was three times that of the cells supplemented with oleic acid. Specific increase in the free fatty acid

TABLE II. Effect of palmitic and oleic acids on lipid composition in V79-R cells. Cells (2.5×10^5) were transferred to a 60 mm tissue-culture dish in DMEM and then grown for 24 h in the presence of palmitic acid or oleic acid (100 μM). Lipids were extracted from cells dispersed with trypsin and assayed as described in "MATERIALS AND METHODS." Values represent the mean of two experiments.

Cell	Lipid class (nmol/10^6 cells)				Cell number (cells $\times 10^{-5}$/dish)
	Phospholipid	Triacylglycerol	Sterol	Free fatty acid	
Control[a]	70.7	12.0	9.3	22.6	3.78
Palmitic acid	153.7	157.1	14.8	118.6	2.10
Oleic acid	51.7	55.0	12.4	13.6	13.00

[a] The cells before the addition of fatty acid.

fraction was observed in the cells supplemented with palmitic acid. The sterol content was specifically unchanged by the addition of palmitic acid.

Effect of fatty acid supplementation on the fatty acid composition of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol

Addition of palmitic acid caused an increase in the proportion of palmitic acid with concomitant decrease of

TABLE III. Effect of palmitic acid on fatty acid composition of cellular lipids. Cells were grown in the presence of palmitic acid as described in Table II. Individual lipid classes were isolated, and their fatty acid compositions were determined as described in "MATERIALS AND METHODS." Values represent the mean of two experiments. "Monoenoic/saturated" refers to the ratio of monoenoic fatty acids to saturated fatty acids.

Fatty acid	PC (wt%)		PE (wt%)		TG (wt%)	
	Control[a]	16 : 0	Control[a]	16 : 0	Control[a]	16 : 0
16 : 0	14.5	30.1	6.7	24.6	11.1	53.1
16 : 1	7.9	13.7	5.1	10.8	5.4	4.5
18 : 0	4.2	7.1	11.4	19.8	9.4	17.9
18 : 1	66.3	44.6	64.8	42.5	46.4	18.5
Others	7.1	4.5	12.0	2.3	27.7	6.0
Class						
Saturated	18.7	37.2	18.1	44.4	20.5	71.0
Monoenoic	74.2	58.3	69.9	53.3	51.8	23.0
Monoenoic/saturated	3.97	1.57	3.86	1.20	2.53	0.32

[a] The cells before the addition of fatty acid.

TABLE IV. Effect of oleic acid on fatty acid composition of cellular lipids. Cells were grown in the presence of oleic acid as described in Table II. Individual lipid classes were isolated, and their fatty acid compositions were determined as described in " MATERIALS AND METHODS." Values represent the mean of two experiments.

Fatty acid	PC (wt%)		PE (wt%)		TG (wt%)	
	Control[a]	18 : 1	Control[a]	18 : 1	Control[a]	18 : 1
16 : 0	14.5	6.7	6.7	5.0	11.1	4.7
16 : 1	7.9	3.0	5.1	1.9	5.4	1.9
18 : 0	4.2	1.9	11.4	9.1	9.4	4.5
18 : 1	66.3	81.0	64.8	68.0	46.4	78.0
Others	7.1	7.4	12.0	16.0	27.7	10.9
Class						
Saturated	18.7	8.6	18.1	14.1	20.5	9.2
Monoenoic	74.2	84.0	69.9	69.9	51.8	79.9
Monoenoic/saturated	3.97	9.77	3.86	4.96	2.53	8.68

[a] The cells before the addition of fatty acid.

oleic acid in phosphatidylcholine and phosphatidylethanolamine (Table III). Thus, the ratio of monoenoic fatty acids to saturated fatty acids in phosphatidylcholine and phosphatidylethanolamine decreased from 3.97 and 3.86 to 1.57 and 1.20, respectively. Addition of oleic acid caused a marked increase of oleic acid in phosphatidylcholine (Table IV). The monoenoic/saturated ratio increased from 3.97 to 9.77. However, the fatty acid composition of phosphatidylethanolamine was not modified by oleic acid. The fatty acid composition of triacylglycerol was greatly modified by the exogenous fatty acids. In the cells supplemented with palmitic acid, more than 70% of the fatty acids in the triacylglycerol were saturated fatty acids, whereas supplementation with oleic acid resulted in similar percentage of unsaturated fatty acids.

Pulse-chase experiments

After 4-h labeling of cells with [1-^{14}C]palmitic acid, about 60% of the total radioactivity incorporated into lipids was recovered in the phosphatidylcholine (Fig. 2A).

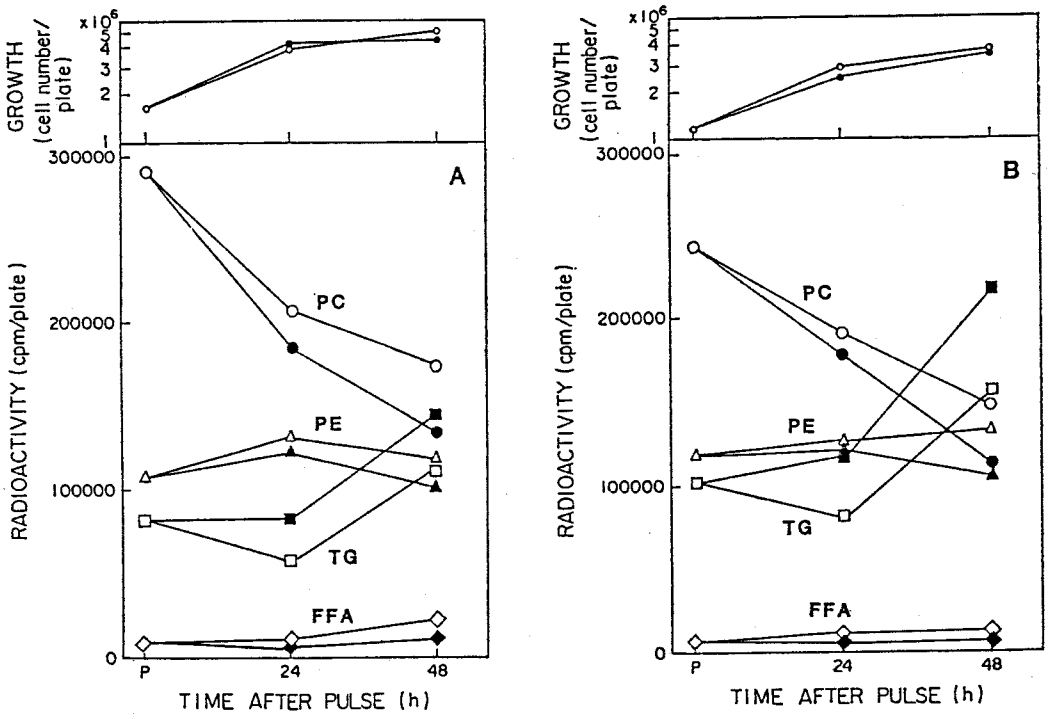

Fig. 2. Turnover of lipids in cells. Cells were labeled for 4 h with [1-^{14}C]palmitic acid (A) or [1-^{14}C]oleic acid (B). Chase was initiated at P. During the chase period, cells were incubated with (solid symbols) or without (open symbols) palmitic acid (A) or oleic acid (B) at 25 μM: PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triacylglycerol; FFA, free fatty acid. Values represent the mean of two dishes.

The phosphatidylethanolamine and triacylglycerol fractions contained about 23% and 17% of the total radioactivity of the lipid fraction. Very small amounts of radioactivity were incorporated into the free fatty acid, monoacylglycerol and diacylglycerol fractions (data not shown). During the

chase period, the radioactivity in phosphatidylcholine decreased rapidly. Radioactivity in the triacylglycerol increased with concomitant decrease in the radioactivity of phosphatidylcholine during the chase period. Radioactivity did not increase in the free fatty acid, monoacylglycerol and diacylglycerol fractions. A similar profile was obtained with [1-^{14}C]oleic acid (Fig. 2B). Cell growth was not inhibited by the addition of 25 μM cold palmitic acid to the medium (Fig. 2A), and the cellular lipids profile in the presence of palmitic or oleic acid remained essentially unchanged (Fig. 2, solid symbols).

Effect of palmitic acid on phospholipid synthesis in endoplasmic reticulum and membrane flow of phospholipid

[2-^{3}H]Glycerol was incorporated in the presence of palmitic acid into the cells prelabeled with [U-^{14}C]leucine. More than 90% of the ^{3}H in both membrane fractions was recovered in phospholipids, not in neutral lipids. The ratios

TABLE V. Synthesis of phospholipid molecular species in endoplasmic reticulum and their membrane flow to plasma membrane. Conditions were described in " MATERIALS AND METHODS." Values in parentheses are expressed as percentages. Ms, crude microsome; Pm, plasma membrane; S-S, phospholipid with two saturated fatty acids; S-M, phospholipid with one saturated and one monoenoic fatty acid; M-M, phospholipid with two monoenoic fatty acids.

Phospholipid molecular species	Control		Palmitic acid	
	^{3}H dpm/^{14}C dpm		^{3}H dpm/^{14}C dpm	
	Ms	Pm	Ms	Pm
Total	4.82 (100)	5.54 (100)	2.67 (100)	4.57 (100)
S-S	0.10 (2)	0.17 (3)	0.14 (5)	0.41 (9)
S-M	2.10 (44)	2.77 (50)	1.81 (68)	2.94 (64)
M-M	2.62 (54)	2.60 (47)	0.72 (27)	1.21 (27)

of ^{3}H in phospholipid to ^{14}C in protein in plasma membrane and microsome fractions are shown in Table V. In the microsome fractions, the $^{3}H/^{14}C$ ratio decreased from 4.82 in control cells to 2.67 in the cells treated with palmitic acid. Following palmitic acid supplementation, the phospholipid ^{3}H/protein ^{14}C ratios for phospholipids with two saturated fatty acids (S-S species) and with one saturated and one monoenoic fatty acid (S-M species) were similar to those in control cells, whereas the ratio for phospholipids with two monoenoic fatty acids (M-M species) was greatly reduced from 2.62 to 0.72. This indicates that the decrease in the $^{3}H/^{14}C$ ratio for the microsome fraction was mainly due to a decrease of M-M species. The phospholipid molecular species composition in the plasma membrane fraction was similar to that in the microsome fraction.

TABLE VI. Synthesis of phospholipid classes and molecular species in endoplasmic reticulum. Conditions were described in " MATERIALS AND METHODS." Abbreviations were shown in Table V.

Phospholipid	Control				Palmitic acid			
	^{3}H dpm/ ^{14}C dpm	Molecular species (%)			^{3}H dpm/ ^{14}C dpm	Molecular species (%)		
		S-S	S-M	M-M		S-S	S-M	M-M
Phosphatidylcholine	2.61	2	51	47	1.16	5	71	24
Phosphatidylethanolamine	0.62	1	54	45	0.49	5	73	22
Phosphatidylinositol	0.50	—	—	—	0.34	—	—	—
Phosphatidylserine	0.19	—	—	—	0.11	—	—	—
Other[a]	0.89	—	—	—	0.57	—	—	—

[a] Material migrating with a mobility greater than that of phosphatidylethanolamine.

The $^{3}H/^{14}C$ ratio of lipid classes in the microsome fraction is shown in Table VI. The ratio decreased significantly in phosphatidylcholine in the presence of palmitic

acid. The proportions of M-M species in phosphatidylcholine and phosphatidylethanolamine were lower in the cells supplemented with palmitic acid. Thus, palmitic acid considerably inhibited the synthesis of phosphatidylcholine and the M-M species of phospholipids. However, membrane flow (29) of phospholipids from endoplasmic reticulum to plasma membrane was preserved.

The molecular species composition of triacylglycerol

The content of triacylglycerol per cell increased significantly when palmitic acid was added to DMEM (Table VII). About 40% of the total radioactivity was localized in tri-

TABLE VII. Synthesis of triacylglycerol molecular species in the presence of palmitic acid. Conditions were described in " MATERIALS AND METHODS." S-S-S, triacylglycerol with three saturated fatty acids; S-S-M, triacylglycerol with two saturated and one monoenoic fatty acids; S-M-M, triacylglycerol with one saturated and two monoenoic fatty acids.

Molecular species	Incorporation of [2-^{3}H]glycerol (%)
S-S-S	28.5
S-S-M	42.4
S-M-M	26.6
Unknon	2.5

acylglycerols with two saturated and one monoenoic fatty acids (S-S-M species). Triacylglycerols with three saturated (S-S-S species) and with one saturated and two monoenoic fatty acids (S-M-M species) held about 29 and 27%, respectively.

Electron microscopy of cells supplemented with palmitic acid

A large amount of triacylglycerol accumulated in the cells supplemented with palmitic acid (Fig. 5). In addition to triacylglycerol accumulation, fiber-like membrane structures were found to be distributed in the cell (Fig. 6). In the presence of oleic acid, triacylglycerol also accumulated, whereas cell division occurred normally and the

TABLE VIII. Mean cell diameter and cell volume. Cells were grown in the presence of palmitic acid and oleic acid as described in Table II. Cell diameter and volume were determined as described in "MATERIALS AND METHODS."

Cell	Diameter (μm)	Volume (μm^3/cell)
Control[a]	16.7	3,806
Palmitic acid	16.2	4,444
Oleic acid	16.4	4,415

[a] The cells before the addition of fatty acid.

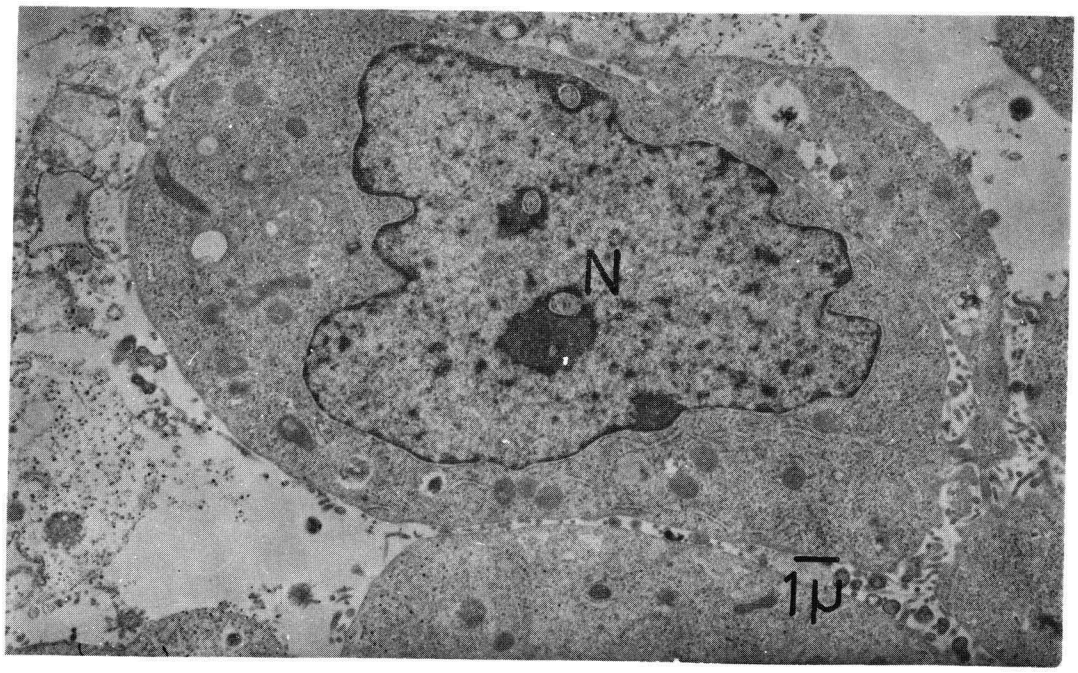

Fig. 3. Electron micrograph of a V79-R cell. N, nucleus. x6,300.

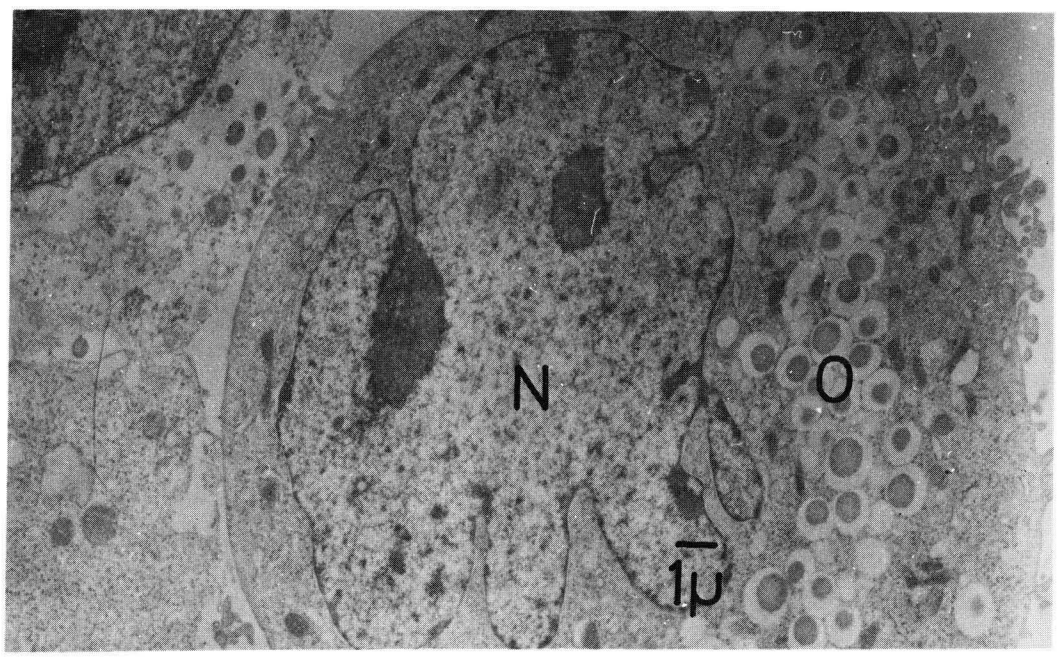

Fig. 4. Electron micrograph of a V79-R cell from culture incubated for 24h in the presence of oleic acid (100 μM). N, nucleus; O,oil body. x6,300.

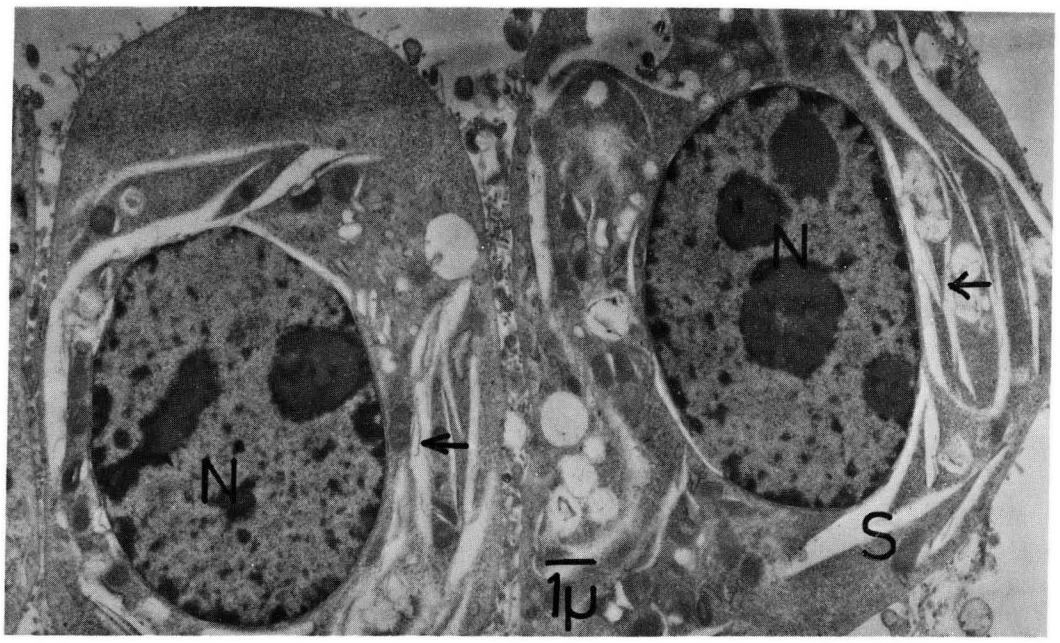

Fig. 5. Electron micrograph of V79-R cells from culture incubated for 24h in the presence of palmitic acid (100 μM). The cell contain cytoplasmic slits (S) and fiber-like membrane structures (arrow). N, nucleus. x6,300.

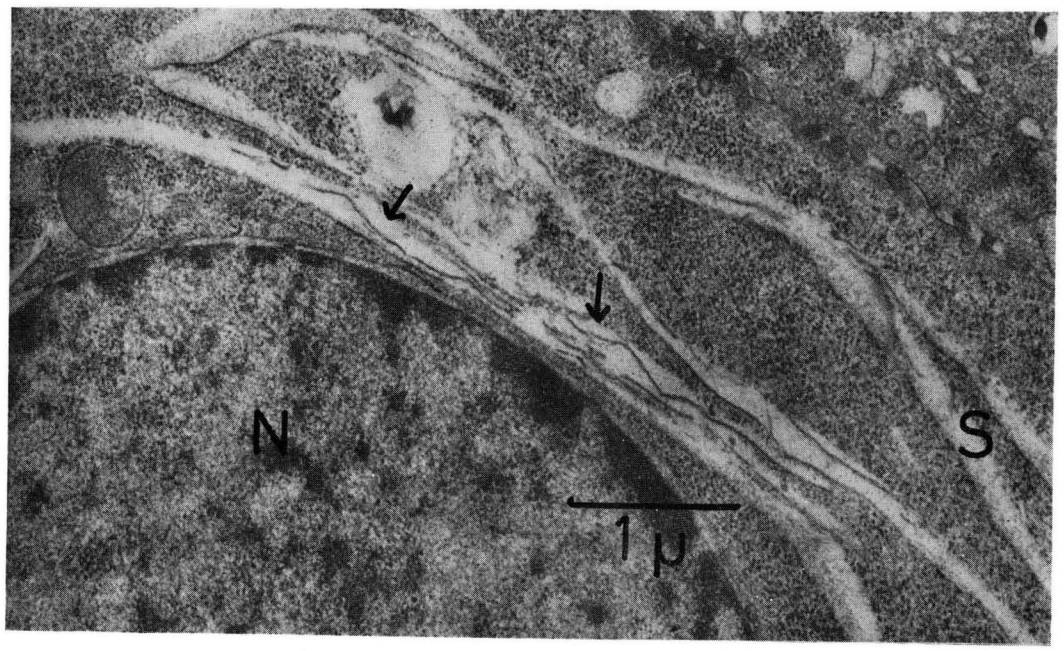

Fig. 6. Electron micrograph of fiber-like membrane structures of V79-R cell from culture incubated for 24h in the presence of palmitic acid (100 μM). Abbreviations are described in Fig. 5. x24,000.

fiber-like membrane structure was not distributed (Fig. 4). However, in the presence of palmitic acid, mean cell diameter and cell volume were unchanged (Table VIII) and the amount of protein and DNA per cell were similar to each other (data not shown).

IV-4 DISCUSSION

I have shown that the addition of saturated fatty acids bound to bovine serum albumin resulted in the inhibition of V79-R cell growth. Saturated fatty acid toxicity has been observed with L cells (57), human skin fibroblasts (56), LM cells (53) and neuroblastoma x glioma hybrid cells (55). In the V79-R cell system, supplementation with short-chain saturated fatty acid (C_8) did not inhibit growth. The effects of saturated fatty acid supplementation on cell growth depended on carbon chain lengths (C_{10}-C_{16}). Among the saturated fatty acids, palmitic acid and myristic acid caused the most severe reduction in growth rate. Kito and Yamamori (58) reported that V79 cell growth was inhibited by the addition of erucic acid (*cis*, $C_{22:1\Delta13}$) or brassidic acid (*trans*, $C_{22:1\Delta13}$), whose physical properties may be similar to those of saturated fatty acids. Thus, the toxic effects of the saturated fatty acids are related to their physical properties. Doi *et al*. (53) demonstrated that the lethal effect of palmitic acid was prevented by the simultaneous addition of unsaturated fatty acid. A similar protec-

tive effect of unsaturated fatty acids was demonstrated in V79-R cells. Experiments with radioactive fatty acids showed that the metabolic fate of palmitic acid was similar to that of oleic acid. However, both triacylglycerol and phospholipid increased when the cells were exposed to palmitic acid. The electron micrographs of the cells supplemented with palmitic acid showed large cytoplasmic clefts which are due to the cylindrical-solid deposits of triacylglycerol. The triacylglycerol in the cells was high in the cells in S-S-S and S-S-M species (Table VII). This indicates that the triacylglycerol synthesized in the presence of palmitic acid was solid and crystalline within the cells at 37°C. The accumulation of the crystallized lipid may perturb cell functions and injure cells. In the presence of oleic acid, only triacylglycerol accumulated. This was stored in the form of oil bodies with diameters of about 1μm (Fig. 4).

Although the synthesis of phospholipids was slightly inhibited in cells with palmitic acid, the phospholipid content per cell increased 2- or 3- fold. In these cells, fiber-like membrane structures were found in the clefts. These structures may be composed of the increased phospholipids. The origin of these membrane structures and their relationship to other organella membranes are still unclear. Moreover, the increased phospholipids may constitute membranes enclosing the cylindrical-solid triacylglycerols in a

monolayer form. However, the amount of phospholipid required to envelop a triacylglycerol droplet (68) is small compared with the amount of triacylglycerol.

Membrane flow from endoplasmic reticulum to plasma membrane was observed both in the cells supplemented with palmitic acid and in control cells (Table V). However, synthesis of the M-M species in the endoplasmic reticulum was specifically inhibited by the addition of palmitic acid. As a results, the proportion of S-M species increased. A decrease in M-M species was also observed in the plasma membrane. The decrease in M-M species may cause significant changes in the physical properties and the physiological functions of the biological membranes.

V LIPID METABOLISM IN CHINESE HAMSTER V79-R MEMBRANES COMPOSED OF UNUSUAL PHOSPHOLIPID MOLECULAR SPECIES

V-1 INTRODUCTION

Most of the enzymes catalyzing the metabolism of phospholipids are closely associated with, or embedded, in cellular organellar membranes. However, interrelationship between composition and metabolism of membrane phospholipids are not yet fully understood.

It has been shown that addition of saturated fatty acid to a medium inhibits animal cell growth (53-58). A similar inhibitory effect of erucic acid (cis, $C_{22:1\Delta13}$) was observed with V79 cells (58). The supplementation of these exogenous fatty acids could alter the acyl residues of membrane phospholipids (53, 55, 56). Previously, I described (chapter IV) that the increases in the ratio of saturated fatty acids in phospholipids by exogenous palmitic acid did not result from an increase in the phospholipid molecular species with two saturated fatty acids, but a decrease in the molecular species with two monoenoic fatty acids. Palmitic acid inhibited synthesis of phospholipids with two monoenoic fatty acids (mainly 1,2-dioleoyl molecular species), and caused an increase in the proportion of phospholipids with one monoenoic and one saturated fatty acid (mainly 1-palmitoyl-2-oleoyl molecular species). Changes in the physical state of the membrane lipids have been shown

to affect several membrane functions (5,6). Modification of membrane phospholipids with saturated fatty acids also has inhibitory effects on the activities of intrinsic membrane enzymes, such as adenylate cyclase and ouabain-sensitive (Na^+, K^+)-ATPase (69), of endocytosis (70) and of amino acid transport (71). However, the interrelationship between the effect of saturated fatty acids on membrane functions and cell growth has not been well characterized.

A number of studies have demonstrated necrosis and fibrosis in the hearts of male rats fed rapeseed oil containing erucic acid (72, 73). Rapeseed oil also induces changes in mitochondrial structure and function (74-76), and inhibits turnover of the 1-stearoyl-2-arachidonoyl phosphatidylcholine in the heart (77, 78). However, the biochemical mechanism of toxicity of erucic acid remains unclear. In this chapter, we used V79-R cells, which can grow in delipidated medium, in an attempt to study the behavior of erucic acid during membrane lipid metabolism. We demonstrate the effect of erucic acid on the composition of cellular phospholipid molecular species, and that the inhibition of phospholipids synthesis in the cells by the addition of erucic acid is associated with the decrease in activity of sn-glycerol 3-phosphate acyltransferase which catalyzes the reaction to form a key intermediate, 1-acyl-sn-glycerol 3-phosphate, for phospholipid synthesis.

V-2 MATERIALS AND METHODS

Materials

[2-^{3}H]Glycerol (10.0 Ci/mmol), sn-[U-^{14}C]glycerol 3-phosphate (144.0 mCi/mmol) and Aquasol-2 were purchased from New England Nuclear Corp., Boston, MA. [U-^{14}C]Leucine (351.0 mCi/mmol) was from Amersham International Limited, Amersham. [1-^{14}C]Erucic acid (40.0 mCi/mmol) was obtained from Centre d'Etudes Nucléaires, Saclay. Fetal bovine serum was from Microbiological Associates Bioproducts, Walkerville, MD. Trypsin (1:250) was from DIFCO Laboratories, Detroit, MI. Fatty acid-free bovine serum albumin was the product of Miles Laboratories Inc., Elkhart, IN. Phospholipase C (*Bacillus cereus*) was from Boehringer Mannheim, Mannheim. Thin-layer plates (No. 5721 and 5724) were purchased from Merck, Darmstadt. Unisil (100-200 mesh) was from Clarkson Chemical Company Inc., Willamsport, PA. All other chemicals were of analytical grade. Palmitoyl-CoA was synthesized according to the method of Seubert (79) and purified by thin-layer chromatography (80) with cellulose plates from Asahi Chemical Industry. The concentration of palmitoyl-CoA solution was determined by assaying the absorbances at 232 and 260 nm (81).

Cells

Chinese hamster V79-R lung fibroblasts (chapter II) were used throughout this study. Cells were routinely maintained in minimum essential Eagle's medium supplemented

with 6% delipidated fetal bovine serum (buffer 1) at 37°C in a humidified atmosphere of 95% air/5% CO_2 in 60 mm Falcon tissue culture dishes. Delipidated serum was prepared according to the method described by Cham and Knowles (21).

Incorporation of [^{3}H]glycerol and [^{14}C]leucine into endoplasmic reticulum and plasma membrane

Cultures were initiated by seeding dishes with 2.5 x 10^5 cells. After 21-h preculture, the cultures were washed twice with 2 ml of Ca^{2+}- and Mg^{2+}-free Hank's solution (buffer 2) and incubated with 4 ml of leucine-free buffer 1 containing [U-^{14}C]leucine (1 μCi). In this experiment, delipidated serum was dialyzed against 67 vol. of buffer 2 at 4°C for 24 h, buffer 2 being changed twice during the dialysis. After 3 h incubation, unlabeled leucine (224 μg/ml) and erucic acid (300 μM) were added to the medium, and the cells were cultured for a further 8 h. Then, [2-^{3}H] glycerol (10 μCi) was added to the culture, and the incubation was continued for 16 h. For control experiment, erucic acid was not added. After the labeling, the cells were washed with 2 ml of 0.15 M NaCl and scraped off with a rubber policeman. Unlabeled carrier cells (8 x 10^7) were added to the labeled cells obtained from 10 dishes. These cells were collected by centrifugation at 100 x g for 5 min and washed twice with ice-cold buffer containing 0.25 M sucrose and 1 mM Tris-HCl, pH 7.2. The cell pellets were resuspended in 1 ml of the same buffer and homogenized in a

Dounce homogenizer (type B pestle) using 20 strokes. Preparations of microsomes and plasma membranes from the cell homogenates were carried out by the method of Baldassare et al. (63). The plasma membrane fraction contained negligible amounts of microsomal marker, NADPH-cytochrome c reductase, and mitochondrial marker, succinate-dependent cytochrome c reductase. The ^{3}H and ^{14}C radioactivities in each membrane fraction were counted in Aquasol-2.

Cellular phospholipid synthesis

Cells (2.5×10^{5}) were plated on a petri dish with 4 ml of buffer 1. After 4-h incubation at 37°C, erucic acid (300 μM) was added to the cells. After the addition of erucic acid, cells were incubated in 1 ml of buffer 1 containing erucic acid (300 μM) and [2-^{3}H]glycerol (10 μCi) for 60 min at the selected time points. After labeling, the radioactivity incorporated into phosphatidylcholine and phosphatidylethanolamine was measured. For control experiment, erucic acid was not added into both media for growth and labeling.

Enzyme assays

Ouabain-sensitive (Na^{+}, K^{+})-ATPase activity was assayed as described by Schimmel et al. (18), with the modification that phosphate was determined by the method of Muszbek et al. (82). NADPH- and succinate-dependent cytochrome c reductase were assayed as described by Sottocasa et al. (65). sn-Glycerol 3-phosphate acyltransferase was assayed

by modification of the method of Monroy et al. (83). Cells (2.5 x 10^5 dish) were transferred to 60 mm culture dishes with 4 ml of buffer 1. After 24-h incubation, erucic acid (300 μM) was added to the medium, and the cells were cultured for 24 h. Control cells were grown in the absence of erucic acid. After the cultivation, the culture dish was washed once with 2 ml of buffer 2 and once with 2 ml of 1 mM EDTA solution in buffer 2. Control cells from 30 dishes and the cells grown with erucic acid from 50 dishes were scraped off with a rubber policeman. The cells were collected by centrifugation, and the cell pellets were than resuspended in cold buffer containing 0.25 M sucrose and 1 mM Tris-HCl, pH 7.2, and homogenized in a Dounce homogenizer. The homogenates were centrifuged at 2200 x g for 10 min. The pellets were resuspended in the same buffer and centrifuged at 750 x g for 10 min. The supernatants from the centrifugation were combined and centrifuged at 100000 x g for 60 min to yield membrane pellets, which were than assayed in a reaction mixture which contained, in a final volume of 100 μl, 100 mM Tris-HCl buffer, pH 7.5, 2mM $MgCl_2$, 1.5 mM sn-[^{14}C] glycerol 3-phosphate (5000 cpm/nmol), 65 μM palmitoyl-CoA, 4% bovine serum albumin and 13 μg protein. The reaction mixture was incubated for 20 s at 37°C prior to the addition of palmitoyl-CoA, and the reaction was continued for 2 min. Then, a 50 μl aliquot of the reaction mixture was taken out and used for the filter paper disk method (84, 85).

Pulse-chase studt

For pulse-chase study, cells were plated on a 60-mm dish with 4 ml of buffer 1. Inoculum density was 3.6 x 10^3 cells/cm^2. After 3-day incubation at 37°C, the medium was removed, and 4 ml fresh buffer 1 containing [1-^{14}C] erucic acid (1 μCi) complexed albumin (29) was added to the cells. After 4-h incubation, the radioactive medium was removed, and cells were washed twice with 2 ml of buffer 2 containing fatty acid-free albumin, and then twice with 2 ml of buffer 1. Nonradioactive buffer 1 (4 ml) containing 25 μM erucic acid was added, and the incubation was continued for 24 and 48 h at 37°C. The cells were dispersed with 0.25% trypsin. The radioactivities of lipid fractions were measured with a Packard liquid scintillation spectrometer in a solution composed of 0.6% PPO (w/v) and 0.05% POPOP (w/v) in toluene.

Lipid analysis

Lipids were extracted from cells by the method of Bligh and Dyer (22). Phospholipids were separated by thin-layer chromatography with a solvent system of chloroform/acetone/methanol/water/acetic acid (100:100:50:10:4, by vol.) for the first development and then with chloroform/methanol/acetic acid/water (180:150:30:10, by vol.) in the same direction. Neutral lipids were separated by thin-layer chromatography using the solvent system chloroform/benzene/methanol (25.5:500:15, by vol.). Lipids were identified by co-chromatography with authentic lipids. Phospholipid

phosphate was measured by the method of Bartlett (59). The amounts of triacylglycerol and of free fatty acid were determined by the method of Biggs et al. (60) and of Novác (61) using triolein and palmitic acid as standards, respectively. For fatty acid analysis, lipids were transmethylated with sodium methoxide, and the fatty acid methyl esters were determined by GLC using a 10% Silar 10C column according to the method of Kito et al. (23). Phospholipid molecular species were determined by the method of Kito et al. (23).

Protein content

Protein was determined by the method of Lowry et al. (28), using bovine serum albumin as standard.

V-3 RESULTS

Effect of erucic acid on growth of V79-R cells

The addition of erucic acid complexed to bovine serum albumin to a medium resulted in inhibition of cell growth (Table I). The effect was dependent on the concentration of erucic acid. At higher concentrations (over 200 μM), the cell number decreased markedly within 48 h when cultures were initiated by seeding 1×10^5 cells on a dish. When the cells were seeded at 2.5×10^5/dish, the growth was inhibited, but the cell number was not reduced, even in the presence of 300 μM erucic acid (data not shown). Oleic acid at 400 μM did not inhibit growth. Therefore, 2.5×10^5 cells

TABLE I

INHIBITION OF CELL GROWTH BY ERUCIC ACID

Cells ($1 \cdot 10^5$) were transferred to a dish with 4 ml buffer 1 and then grown in the presence of erucic acid. The number of cells was determined with a hemocytometer. Values represent the mean of two dishes.

Concentration (μM)	Cell growth (cells ($\cdot 10^{-5}$)/dish)		
	Incubation time (h)		
	0	24	48
None	1.05	1.85	5.80
50		1.85	3.90
100		1.45	3.50
200		0.96	0.54
400		0.23	0
500		0	0

were used for the experiments, except in pulse-chase experiments, in the presence of 300 μM erucic acid.

Effect of erucic acid on cellular lipid compositions

The amount of phospholipids per cell was slightly increased in the presence of erucic acid compared with control cells, whereas the amount of triacylglycerol per cell was increased to about seven times that of control cells (Table II). Erucic acid was rapidly incorporated into the cellular phospholipids (Fig. 1). Erucic acid comprised 53.4 and 28.3 % of the fatty acids of phosphatidylcholine and phosphatidylethanolamine, respectively (Table III). The incorpo-

TABLE II

EFFECT OF ERUCIC ACID ON LIPID COMPOSITION IN V79-R CELLS

Cells ($2.5 \cdot 10^5$) were transferred to a dish with 4 ml buffer 1 and then grown for 24 h in the presence of erucic acid (300 μM). Lipid analyses were carried out as described in Materials and Methods. Values represent the mean of two experiments. Figures in parentheses are nmol/dish.

Addition	Lipid class (nmol/10^6 cells)		
	Phospholipid	Triacylglycerol	Free fatty acid
None	66.6(12.3)	12.0(2.2)	22.4(4.1)
Erucic acid	79.1 (8.3)	84.7(8.9)	46.6(4.4)

ration of erucic acid into the cells caused a small increase in gondoic acid in phospholipids. This suggests that decarboxylation of erucic acid proceeded very slowly.

Canges in phospholipid molecular species composition by erucic acid

The compositions of phosphatidylcholine and phosphatidylethanolamine molecular species in control cells and in cells supplemented with erucic acid are shown in Table IV. The major molecular species in phosphatidylcholine in control cells were the palmitoyl (16:0)/oleoyl (18:1) species (32.6%) and 18:1/18:1 species (42.5%). The

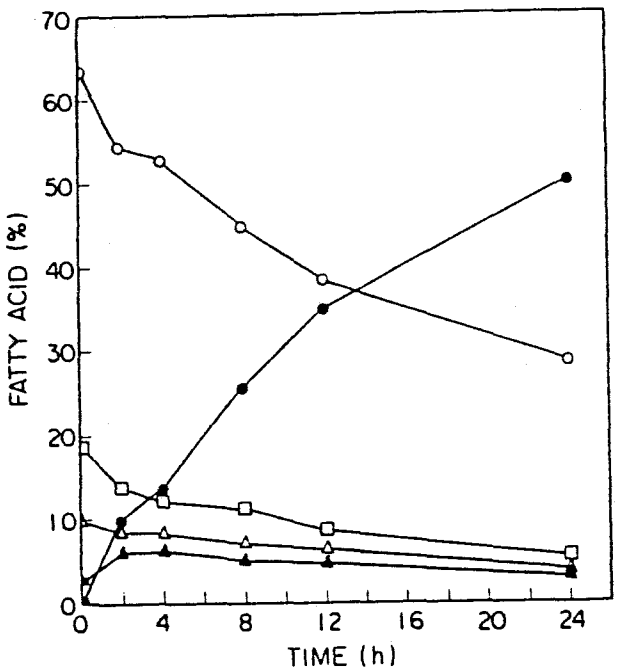

Fig. 1. Incorporation of erucic acid into cellular phospholipids. Cells ($2.5 \cdot 10^5$) were transferred to a dish with buffer 1 and grown further. After 24-h incubation, erucic acid (300 μM) was added to a medium. Phospholipids were isolated from cells, and their fatty acid compositions were determined as described in Materials and Methods. Oleic acid, ○; erucic acid, ●; palmitic acid, □; stearic acid, ▲; palmitoleic acid, △.

TABLE III

EFFECT OF ERUCIC ACID ON FATTY ACID COMPOSITION OF CELLULAR LIPIDS

Cells were grown as described in Table II. Fatty acid compositions were determined as described in Materials and Methods. 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 20:1, gondoic acid; 22:1, erucic acid. Values represent the mean wt.% of total fatty acids of two experiments.

Fatty acid	Phosphatidyl-choline		Phosphatidyl-ethanolamine		Triacylglycerol	
	None	22:1	None	22:1	None	22:1
16:0	18.1	6.2	10.5	10.7	14.7	2.9
16:1	9.3	3.4	6.2	2.9	5.7	0.6
18:0	2.5	1.3	10.9	13.0	6.0	0.6
18:1	64.2	27.7	59.9	34.5	55.6	9.4
20:1	1.8	4.2	1.6	4.4	4.5	2.2
22:1	–	53.4	–	28.3	–	80.4
Not identified	4.1	3.8	10.9	6.2	13.5	3.9

TABLE IV

COMPOSITION OF PHOSPHOLIPID MOLECULAR SPECIES IN V79-R CELLS SUPPLEMENTED WITH ERUCIC ACID

Cells were grown in the presence of erucic acid as described in Table II. Phospholipid molecular species were determined as described in Materials and Methods. S-S, phospholipid with two saturated fatty acids; S-M, phospholipid with one saturated and one monoenoic fatty acid; M-M, phospholipid with two monoenoic fatty acids; E-M, phospholipid with one erucic and one monoenoic fatty acid; E-E, phospholipid with two erucic acids. Values represent the mean wt.% of total molecular species of two experiments.

Molecular species	None		22:1	
	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-choline	Phosphatidyl-ethanolamine
S-S				
16:0/16:0	0.6	-	0.1	0.3
S-M				
16:0/16:1	5.3	0.8	0.4	0.6
16:0/18:1	32.6	26.1	4.2	10.7
18:0/18:1	3.3	27.1	1.3	9.4
Not identified	-	1.7	1.4	2.8
M-M				
16:1/16:1	0.6	-	0.3	6.6
18:1/16:1	12.3	4.6	1.7	11.5
18:1/18:1	42.5	36.0	5.0	13.4
E-M				
22:1/18:1	-	-	36.1	17.8
22:1/20:1	-	-	6.7	8.0
E-E				
22:1/22:1	-	-	36.6	10.5
Not identified	2.8	3.7	6.2	8.3

major molecular species in phosphatidylethanolamine were the 16:0/18:1 species (26.1%), stearoyl (18:0)/18:1 species (27.1%) and 18:1/18:1 species (36.0%). These phospholipid molecular species compositions were consistent with the fatty acid composition of phospholipids (Table III). By the incorporation of erucic acid into phospholipids, new phospholipid molecular species were formed. The new phosphatidylcholine and phosphatidylethanolamine molecular species were identified to be the erucoyl (22:1)/18:1 species, 22:1/gondoyl (20:1) species and 22:1/22:1 species. This suggests that erucic acid is incorporated into both C-1 and C-2 positions of phospholipids. The ratio of the molecular species containing erucic acid was higher in phos-

phatidylcholine (about 80%) than in phosphatidylethanolamine (about 40%). The increases in the molecular species containing erucic acid resulted in concomitant decreases in the preexisting molecular species.

Effect of erucic acid on phospholipid biosynthesis in endoplasmic reticulum and membrane flow to plasma membrane

The enzyme system for synthesis of major phospholipids in animal cells is localized in endoplasmic reticulum (86). [2-^{3}H]Glycerol was incorporated into microsomal and plasma membrane phospholipid in the absence or presence of erucic acid in cells prelabeled with [U-^{14}C]leucine. More than 90% of the ^{3}H in both membrane fractions was recovered in phospholipids. Though it was not confirmed whether [U-^{14}C] leucine in proteins were the same during the chase in the presence and absence of erucic acid, the ratio of dpm of ^{3}H in phospholipid to dpm of ^{14}C in protein in microsome fraction decreased from 3.13 in control cells to 1.21 in the cells with erucic acid (Table V). This suggests that phos-

TABLE V

SYNTHESIS OF PHOSPHOLIPID CLASSES IN ENDOPLASMIC RETICULUM AND THEIR MEMBRANE FLOW TO PLASMA MEMBRANE IN THE PRESENCE OF ERUCIC ACID

Conditions are described in Materials and Methods. Values represent ratios of ^{3}H in phospholipids to ^{14}C in protein.

Phospholipid	None		22:1	
	Microsome	Plasma membrane	Microsome	Plasma membrane
Total	3.13	5.37	1.21	2.08
Phosphatidylcholine	1.78	2.52	0.60	0.73
Phosphatidylethanolamine	0.28	0.55	0.19	0.31
Phosphatidylserine	0.15	0.46	0.09	0.24
Phosphatidylinositol	0.42	0.88	0.13	0.36
Not identified	0.50	0.96	0.20	0.44

pholipid biosynthesis in endoplasmic reticulum was reduced in the presence of erucic acid. The inhibition is supported by the results shown in Fig. 2.

The major molecular species of phosphatidylcholine and phosphatidylethanolamine, which were synthesized in the presence of erucic acid (Table VII). The 22:1/22:1 molecular species comprised 50 and 44% of total molecular species of phosphatidylcholine and phosphatidylethanolamine,

TABLE VI

SYNTHESIS OF PHOSPHOLIPID MOLECULAR SPECIES IN ENDOPLASMIC RETICULUM AND THEIR MEMBRANE FLOW TO PLASMA MEMBRANE IN V79-R CELLS

Phospholipid molecular species were separated by silver nitrate thin-layer chromatography and the radioactivity of ^{3}H in each spot on plate was measured. Conditions are described in Materials and Methods. Values represent ratios of ^{3}H in phospholipid molecular species to ^{14}C in protein. Values in parentheses represent % distribution of total radioactivity.

Fraction	Microsome		Plasma membrane	
	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-choline	Phosphatidyl-ethanolamine
S-S [a]	0.04 (3)	0.01 (3)	0.20 (5)	0.03 (6)
S-M [b]	0.65(36)	0.12(42)	0.97(43)	0.27(49)
M-M [c]	1.09(61)	0.15(55)	1.35(51)	0.25(46)

[a] This fraction contained 16:0/16:0.
[b] This fraction contained 16:0/18:1, 16:0/16:1 and 18:0/18:1.
[c] This fraction contained 18:1/18:1, 18:1/16:1 and 16:1/16:1.

TABLE VII

SYNTHESIS OF PHOSPHOLIPID MOLECULAR SPECIES IN ENDOPLASMIC RETICULUM AND THEIR MEMBRANE FLOW TO PLASMA MEMBRANE IN THE PRESENCE OF ERUCIC ACID

Phospholipid molecular species were separated by silver nitrate thin-layer chromatogrpahy and the radioactivity of ^{3}H in each spot on the plate was measured. Conditions are described in Materials and Methods. Values represent ratios of ^{3}H in phospholipid molecular species to ^{14}C in protein. Values in parentheses represent % distribution of total radioactivity.

Fraction	Microsome		Plasma membrane	
	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-choline	Phosphatidyl-ethanolamine
S-S [a]	0.01 (2)	0.01 (4)	0.04 (5)	0.03(10)
S-M [b]	0.04 (7)	0.03(17)	0.12(17)	0.07(22)
M-M + M-E [c]	0.25(41)	0.07(34)	0.35(48)	0.14(46)
E-E [d]	0.30(50)	0.08(44)	0.22(30)	0.31(22)

[a] This fraction contained 16:0/16:0.
[b] This fraction contained 16:0/18:1, 16:0/16:1 and 18:0/18:1.
[c] This fraction contained 18:1/18:1, 18:1/16:1, 16:1/16:1, 22:1/20:1 and 22:1/18:1.
[d] This fraction contained 22:1/22:1.

respectively, which had been newly synthesized. Most of the remaining radioactivity was found in the 22:1/18:1 and 18:1/18:1 species. However, the major molecular species of phosphatidylcholine and phosphatidylethanolamine synthesized in the control cells were 16:0/18:1 and 18:1/18:1 species, respectively (Table VI).

The phospholipids synthesized in endoplasmic reticulum are known to be transported to other organellar membranes, including plasma membrane, through the process referred to as " membrane flow" (67). In order to check whether the flow of membrane phospholipid is affected by the addition of erucic acid, the $^{3}H/^{14}C$ ratios in the microsome and plasma membrane fractions from the cells supplemented with erucic acid were compared with those from the control cells (Table V). The ratio of the $^{3}H/^{14}C$ in the plasma membrane fraction against the sum of the ratios of $^{3}H/^{14}C$ in the microsome and plasma membrane fractions shows the extent of transport of phospholipids from endoplasmic reticulum to plasma membrane. There was little difference in the ratio between the control cells and erucic acid-treated cells (0.62: 0.63). This suggests that erucic acid has no effect on membrane flow of phospholipids from endoplasmic reticulum to plasma membrane. The composition of molecular species of phospholipids transported to plasma membrane reflected the composition of phospholipid molecular species synthesized in endoplasmic reticulum (Table VI and VII). The fact that plasma membranes

have less E-E and more (M-M, M-E) molecular species than the microsomal fractions may be due to differences in transport rate from microsome among individual molecular species.

Effect of erucic acis on cellular phospholipid synthesis and sn-glycerol 3-phosphate acyltransferase activity

As erucic acid was incorporated into phospholipids (Fig. 1), phospholipid synthetic activity of the cell was reduced (Fig. 2). Incorporation of [^{3}H]glycerol into phos-

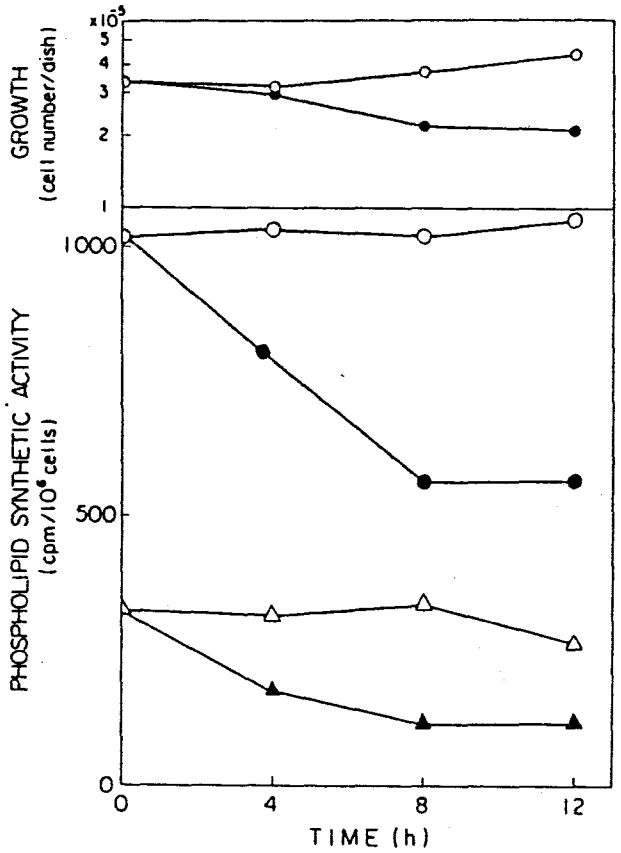

Fig. 2. Cellular phospholipid synthesis in the presence or absence of erucic acid. Conditions are described in Materials and Methods. [2-^{3}H]Glycerol incorporation was measured. Values represent the mean of two experiments. Phosphatidylcholine with erucic acid, ●; without erucic acid, ○; phosphatidylethanolamine with erucic acid, ▲; without erucic acid, △.

phatidylcholine and phosphatidylethanolamine for 60 min after 8 h incubation in the presence of erucic acid decreased to about 60 and 40%, respectively, of that of control cells.

The amount of phospholipids synthesized in endoplasmic reticulum was reduced by the addition of erucic acid (Table V). The specific activity of sn-glycerol 3-phosphate acyltransferase which catalyzes the reaction to form a key intermediate, 1-acyl-sn-glycerol 3-phosphate, in biosynthesis of phospholipids (68) in the membrane fraction from the cells supplemented with erucic acid was about 64% of that from the control cells (2.82 $\pm$ 0.14 and 1.81 $\pm$ 0.03 nmol/min per mg protein for the enzyme from the cells grown in the absence and presence, respectively, of erucic acid, $P < 0.001$). Thus, the reduction of the synthesis of phospholipids may be attributable to the decrease in sn-glycerol 3-phosphate acyltransferase activity.

Turnover of erucic acid incorporated into cellular lipids

The composition of phosphatidylcholine molecular species was altered more than that of phosphatidylethanolamine molecular species when the cells were supplemented with erucic acid (Table IV). The difference may be due to the difference in turnover rate of phosphatidylcholine and phosphatidylethanolamine. Radioactive erucic acid was predominantly incorporated into phosphatidylcholine, phosphatidylethanolamine and triacylglycerol (Fig.3A and B). During the

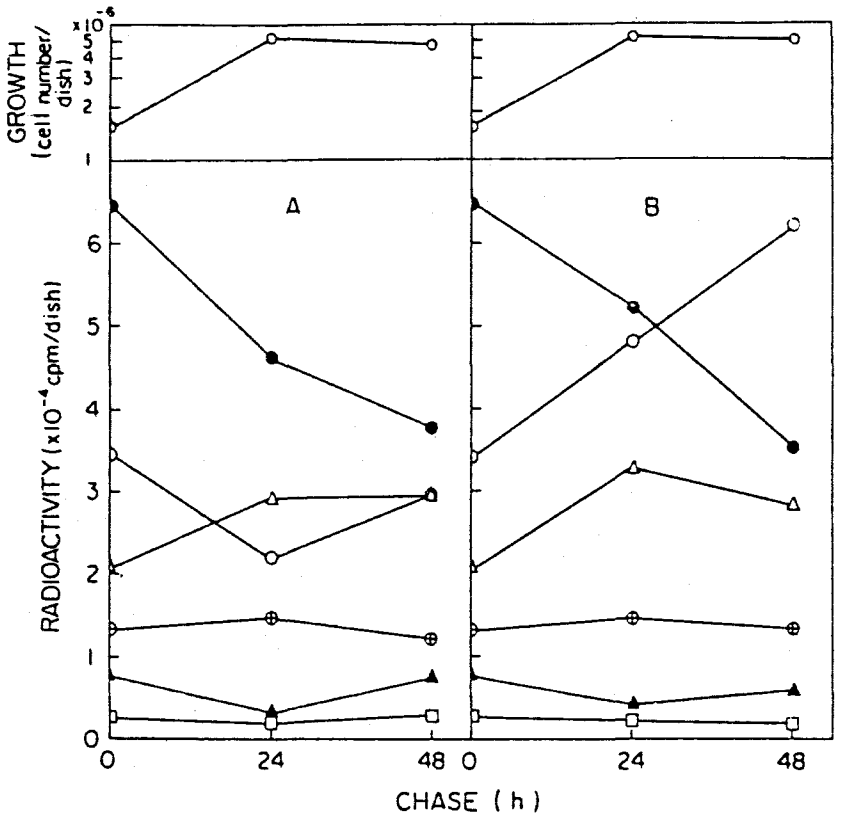

Fig. 3. Chase of lipids labeled with [1-^{14}C]erucic acid. Conditions are described in Materials and Methods. Values represent the mean of two experiments. A, Without; B, with 25 μM erucic acid in the medium during chase. Phosphatidylcholine, ●; triacylglycerol, ○; phosphatidylethanolamine, △; free fatty acid, ⊕; diacylglycerol, ▲; monoacylglycerol, □.

chase period, the radioactivity in phosphatidylcholine decreased rapidly, with a concomitant increase in radioactivity in triacylglycerol in the presence of erucic acid. Little change in the radioactivity in phosphatidylethanolamine was observed during the chase. The profile obtained with [1-^{14}C]erucic acid was basically similar to that with [1-^{14}C]palmitic acid or [1-^{14}C]oleic acid reported previously (chapter IV).

V-4 DISCUSSION

We have shown the relationship between the inhibition of animal cell growth and the changes in lipid metabolism caused by erucic acid. The results indicate three characteristic effects of erucic acid on V79-R cells: (1) formation of unusual phospholipid molecular species containing erucic acid, (2) inhibition of phospholipid synthesis in endoplasmic reticulum, (3) reduction of sn-glycerol 3-phosphate acyltransferase activity.

The amount of phospholipids per cell increased when the cell growth was inhibited in the presence of erucic acid (Table II). This may be due to the difference in the extent of inhibition by erucic acid between cell division and phospholipid synthesis. A number of studies have indicated that membrane phospholipids are modified by exogenous fatty acid (53-55). However, little is known about changes in phospholipid molecular species composition. The incorporation of erucic acid into phospholipids resulted in the formation of 22:1/18:1 and 22:1/22:1 molecular species. The proportion of molecular species containing erucic acid was higher in phosphatidylcholine than in phosphatidylethanolamine. This may be due to the faster turnover rate of phosphatidylcholine than of phosphatidylethanolamine.

Since the inhibition of phospholipid synthesis in endoplasmic reticulum by erucic acid was nonspecific to phospholipid classes, we determined the activity of sn-glycerol

3-phosphate acyltransferase, which formes a key intermediate, 1-acyl-sn-glycerol 3-phosphate, for phospholipid synthesis. The specific activity of the enzyme in the membrane fraction isolated from cells supplemented with erucic acid was lower than that from the control cells. Inhibition of the total biosynthesis of phospholipids may be attributed to a decrease in the specific activity of sn-glycerol 3-phosphate acyltransferase. To explain the lower specific activity of the enzyme in the cells supplemented with erucic acid, the following possibilities are assumed. (a) Erucic acid may specifically decrease the synthesis of the sole acyltransferase protein or increase the degradation of the sole acyltransferase. At present there is no evidence to support this possibility. (b) Phospholipid(s) which is essential for the enzyme activity may be reduced by erucic acid. sn-Glycerol 3-phosphate acyltransferase solubilized from Escherichia coli B membrane is highly activated by phosphatidylglycerol (87). sn-Glycerol 3-phosphate acyltransferase extracted from rat liver mitochondria also requires phospholipids (88). However, it is unlikely that the essential phospholipid class(es) for enzyme activity is reduced, since distribution of the polar head group composition of phospholipids was not influenced by erucic acid. However, we can not exclude the possibility that some phospholipid molecular species, which is essential to activate the enzyme, is not fully supplied for the en-

zyme, since new phospholipid molecular species of 22:1/18:1 and 22:1/22:1 were increased by erucic acid. (c) Erucic acid has a higher melting point (34.7°C) compared to oleic acid (13.4-16.3°C), which is a major fatty acid in the cellular phospholipids. Changes in physical properties of the membrane by the incorporation of significant amounts of erucic acid into the phospholipids may decrease sn-glycerol 3-phosphate acyltransferase activity. Silbert and co-workers (89, 90) have shown the regulatory mechanism to maintain membrane fluidity by changing the ratio of oleic and palmitic acids and by changing the ratio of phosphatidyl-choline and phosphatidylethanolamine in responce to sterol depletion in LM cells. However, the incorporation of erucic acid into cellular membranes did not induce any compensatory changes in phospholipid polar head group composition, nor changes in sterol content. This suggests that membrane fluidity may be altered by an increase of erucic acid in phospholipids. Generally, membrane functions are known to be affected by changes in membrane fluidity (5, 6, 91, 92).

Triacylglycerol was accumulated in the presence of erucic acid (Table II), despite the decrease in the sn-glycerol 3-phosphate acyltransferase activity, which may affect triacylglycerol formation. During the chase period, almost all of the radioactivity of phosphatidylcholine, the fatty acyl chains of which had been labeled with [1-^{14}C] erucic acid, was recovered in triacylglycerol in the pres-

ence of erucic acid in the medium, whereas the radioactivity was recovered only partially in triacylglycerol in the absence of erucic acid in the medium (Fig. 3A and B). Thus, the diacylglycerol moiety of phosphatidylcholine may be precursor of triacylglycerol. In this case, the pathway of triacylglycerol formation may not pass through free fatty acid, since unlabeled erucic acid did not dilute the radioactivity of erucic acid released from phosphatidylcholine. However, in order to elucidate the inhibitory mechanism by erucic acid, it may be necessary to examine the effect of erucic acid on enzymes of phospholipid synthesis, other than sn-glycerol 3-phosphate acyltransferase.

The pathological lesions of myocardium in male rats caused by dietary rapeseed oil containing erucic acid have been reported by several investigators (72, 73). Kito and co-workers (77, 78) have suggested that these fatal injuries may results from inhibition of metabolism of 1-stearoyl-2-arachidonoyl phosphatidylcholine in rat heart by erucic acid. However, another mechanism must be proposed to explain the inhibition of V79-R cell growth by erucic acid, since the phospholipids in the cells contain little arachidonic acid. There is a possibility that the inhibition of phospholipid synthesis by erucic acid reduced the supply of the specific phospholipid class essential for cell division. Palmitic acid inhibited cell growth more severely than did erucic acid, though the extent of in-

hibition of phospholipid synthesis by palmitic acid was weaker than that by erucic acid and phospholipid content in the cells supplemented with palmitic acid was twice that in control cells (chapter IV). This may exclude the possibility mentioned above. Besides, normal membrane flow of phospholipids from endoplasmic reticulum to cytoplasmic membrane in the presence of erucic acid may eliminate the cause of reduced supply of phospholipids for cytoplasmic membrane. An increase in the molecular species containing erucic acid in membrane phospholipids may change membrane fluidity and disturb membrane function. Some enzyme activities necessary to cell division are assumed to be inhibited in the membranes composed of unusual phospholipid molecular species.

VI PHOSPHOLIPID METABBOLISM IN V79-R MEMBRANE COMPOSED OF PHOSPHOLIPID MOLECULAR SPECIES CONTAINING trans-MONOENOIC FATTY ACID

VI-1 INTRODUCTION

The interrelationship between the components and physiological functions of membrane is not yet fully understood. Several techniques to alter the lipid composition of animal membranes have been established. Modification of phospholipid polar head groups or acyl chains has been achieved by the addition of base analogs (93) or fatty acids complexed to bovine serum albumin (54,94). Alteration of sterol content has also been reported (95). These techniques have been applied to elucidate the interrelationship between membrane structure and its functions such as enzymes (69,91,92), transport (71), and endocytosis (70).

Previously, we described (chapter IV) that exogenous palmitic acid (16:0) specifically inhibited synthesis of cis-octadecenoic acid(cis-18:1)/cis-18:1 molecular species, which are major molecular species in normal V79-R membranes. Erucic acid (cis-13-22:1) added to the medium resulted in formation of new phospholipid molecular species containing cis-13-22:1 and inhibited de novo synthesis of phospholipids (chapter V). Cell growth was inhibited by these fatty acids. Trans-unsaturated fatty acids are produced during partial hydrogenation of vegetable and marine oils.

Ingested trans-fatty acids are incorporated into lipids of body tissues of humans (96,97) and experimental animals (52,98,99). Many studies have demonstrated that feeding rats with diets containing partially hydrogenated oil disturbs the metabolism of essential fatty acids (100, 101).

This chapter describes the effects of trans-monoenoic fatty acids on the composition of membrane phospholipid molecular species and the activities of some membrane-bound phospholipid synthesizing enzymes of V79-R cells, which can grow in delipidated medium.

VI-2 MATERIALS AND METHODS

Materials

CDP[1,2-^{14}C]ethanolamine (97 mCi/mmol) was purchased from ICN Pharmaceuticals, Inc., Irvine, CA. CDP[methyl-^{14}C]choline (52 mCi/mmol) and [methyl-^{3}H]thymidine (25 Ci/mmol) were from Amersham International plc, Amersham, Buckinghamshire, England. sn-[U-^{14}C]Glycerol 3-phosphate (144 mCi/mmol), [methyl-^{14}C]phosphorylcholine (55 mCi/mmol), and Aquasol-2 were obtained from New England Nuclear Corp., Boston, MA. Fetal bovine serum was from Microbiological Associates Bioproducts, Walkerville, MD. Trypsin (1:250) was from DIFCO Laboratories, Detroit, MI. Fatty acid-free bovine serum albumin was the product of Miles Laboratories Inc., Elkhart, IN. Phospholipase C (Bacillus cereus) was from Boehringer Mannheim, Mannheim. Thin-layer plates (No.

5721 and 5724) were purchased from Merck, Darmstadt. All other chemicals were of analytical grade. Palmitoyl-CoA was synthesized according to the method of Seubert (79) and purified by thin-layer chromatography (80) with cellulose plates from Asahi Chemicals Industry. The concentration of palmitoyl-CoA solution was determined by assaying the absorbances at 232 and 260 nm (81).

Cells

Chinese hamster V79-R fibroblasts were used throughout this study. Cells were maintained in minimum Eagle's medium supplemented with 6% delipidated fetal bovine serum at 37°C in a humidified atmosphere of 95% air/ 5% CO_2. Delipidated serum was prepared according to the method described by Cham and Knowles (21). In the experiments, cultures were initiated by seeding dishes with 8.8 x 10^3cells/cm^2. After 24-h preculture, trans-monoenoic fatty acids complexed to bovine serum albumin (29) were added to the cells. The mole ratio of fatty acids to albumin was fifteen. In the control experiment, the same volume of bovine serum albumin solution without trans-monoenoic fatty acid was added.

Lipid analysis

Lipids were extracted from cells by the method of Bligh and Dyer (22). Phospholipids were separated by thin-layer chromatography as previously described (chapter V). Phospholipid phosphate in extracts and separated subfractions on thin-layer plates were measured by the method of Bartlett

(59) and Keenan et al. (102). For fatty acid analysis, lipids were transmethylated with sodium methoxide, and the fatty acid methyl esters were determined by isothermal analysis using a Shimadzu 7A gas chromatograph equipped with a 50 m x 0.25 mm flexible glass capillary column coated with SS-10 (Shinwa Kako Co., Ltd., Kyoto, Japan) at 220°C. Phospholipid molecular species were determined by the method of Kito et al.(23).

Preparations of membrane fragments

Preparation of glycerophosphate acyltransferase was performed as previously described (chapter V). Microsomes were prepared by the method of Coleman and Haynes (103). Microsomal preparations were resuspended in 10 mM Tris-HCl buffer (pH 7.5).

Enzyme assays

Glycerophosphate acyltransferase [EC 2.3.1.15] was assayed as reported previously (chapter V). In this experiment, the activities showed higher values than that of previous assay (chapter V) since the diameter of the reaction tubes was smaller (104). Cholinephosphotransferase [EC 2.7.8.2] and ethanolaminephosphotransferase [EC 2.7.8.1] activities were measured by the method of Coleman and Bell (105) in an incubation mixture containing 175 mM Tris-HCl buffer (pH 8.1), 8 mM $MgCl_2$, 1 mg/ml fatty acid-free bovine serum albumin, 0.1 mM diolein, 20 μM [^{14}C]CDP-choline (52 mCi/mmol) or [^{14}C]CDP-ethanolamine (53 mCi/mmol), 0.5 mM

EGTA, and microsome (20-100 μg protein) in a final volume 100 μl. The reaction was continued for 2.5 min at 25°C. Products were extracted with chroloform/methanol (2:1, by vol.). Radioactivities were measured with a Packard liquid scintillation spectrometer in a solution composed of 0.6% PPO (w/v) and 0.05% POPOP (w/v) in toluene. Almost all the radioactivity (up to 99%) in the extracts from both the reaction mixtures for cholinephosphotransferase and ethanolaminephosphotransferase was recovered in phosphatidylcholine and phosphatidylethanolamine, respectively. Cholinephosphate cytidylyltransferase [EC 2.7.7.15] was assayed by the method of Sleight and Kent (106). The reaction mixture contained 20 mM Tris-succinate buffer (pH 7.8), 6 mM $MgCl_2$, 5 mM CTP, 4 mM [^{14}C]phosphorylcholine (2.5 mCi/mmol), and microsome (20-80 μg protein) in a final volume 50 μl. The tubes were incubated for 20 min at 37°C. The CDP-choline produced was bound to acid-washed charcoal. The charcoal washed with water was then suspended in 0.5 ml of 10% acetic acid and mixed with 5 ml of Aquasol-2 to count the radioactivity.

Protein content

Protein was determined by the method of Lowry et al. (28), with bovine serum albumin as the standard.

VI-3 RESULTS

Effect of trans-monoenoic fatty acids on growth of V79-R

cells

Trans-monoenoic fatty acids added to a medium resulted in inhibition of cell growth (Table I). The extent of the inhibition was dependent on the concentration of trans-monoenoic fatty acids. Though growth was inhibited, the cell number was not reduced for 24 h after addition of 300 μM elaidic acid (trans-9-18:1), trans-11-eicosenoic acid (trans-11-20:1), or 400 μM brassidic acid (trans-13-22:1). These cells were confirmed all viable by Trypan Blue dye inclusion and incorporation of [methyl-^{3}H]thymidine into the cellular DNA. However, oleic acid (cis-9-18:1) at 400 μM did not inhibit growth (data not shown). This indicates that the inhibition was not caused by any surfactant-like property of

TABLE I. Inhibition of cell growth by *trans*-monoenoic fatty acids. Cells (2.5×10^5) in a 60 mm dish with 4 ml Eagle's medium supplemented with 6% delipidated fetal bovine serum were grown in the presence of *trans*-monoenoic fatty acids for 24 h as described in " MATERIALS AND METHODS." The number of cells was determined with a hemocytometer. The medium in each dish was replaced by the *trans*-fatty acid-free medium supplemented with [methyl-^{3}H]thymidine (1 μCi/ml). After incubation for 1 h, cells were precipitated with 5% trichloroacetic acid and used for determination of radioactivity. Values represent the mean of two dishes.

Fatty acid	Concentration (μM)	Cell growth (cell × 10^5/dish)		DNA synthesis 24 h after addition (cpm/mg protein)
		0 time	24 h after addition	
None		2.80	5.60	2,326
Trans-9-18 : 1	100		5.27	
	200		3.55	
	300		2.63	1,449
	400		1.80	
Trans-11-20 : 1	100		5.44	
	200		3.37	
	300		2.40	1,907
	400		2.06	
Trans-13-22 : 1	100		3.82	
	200		3.35	
	300		2.64	
	400		2.06	1,890

the fatty acids. Hence, 300 μM trans-9-18:1 or trans-11-20:1 or 400 μM trans-13-22:1, were used for all experiments.

Effect of trans-monoenoic fatty acids on cellular phospholipid composition

The amount of phospholipids per cell was increased in the presence of trans-9-18:1, trans-11-20:1, and trans-13-22:1. No difference in the polar head group composition of phospholipids was found among the cells supplemented with various trans-monoenoic fatty acids (Table II).

TABLE II. Effect of *trans*-monoenoic fatty acids on phospholipid composition in V79-R cells. Cells were grown in the presence of 300 μM *trans*-9-18 : 1, *trans*-11-20 : 1, or 400 μM *trans*-13-22 : 1 for 24 h as described in Table I. Lipid analyses were carried out as described in " MATERIALS AND METHODS." Numerals in parentheses are nmol phospholipid per mg protein. Values represent the mean of two experiments.

Phospholipid	Addition			
	None	*Trans*-9-18 : 1	*Trans*-11-20 : 1	*Trans*-13-22 : 1
	nmol/10^6 cells			
Total	66.1 (182.6)	90.8 (209.2)	102.7 (221.8)	81.9 (195.0)
	%			
Phosphatidylcholine	50.1	55.9	52.7	44.9
Phosphatidylethanolamine	20.7	19.9	17.6	26.6
Phosphatidylinositol	6.7	7.0	6.6	9.1
Phosphatidylserine	7.0	2.6	5.5	4.2
Sphingomyelin	2.9	—	—	—

Effect of trans-monoenoic fatty acids on the fatty acid composition of phospholipids

Each trans-monoenoic fatty acid was rapidly incorporated into the cellular phospholipids (Fig. 1). After 24-h incubation with trans-9-18:1, trans-11-20:1, and trans-13 22:1, trans-monoenoic fatty acids comprised about 60, 45,

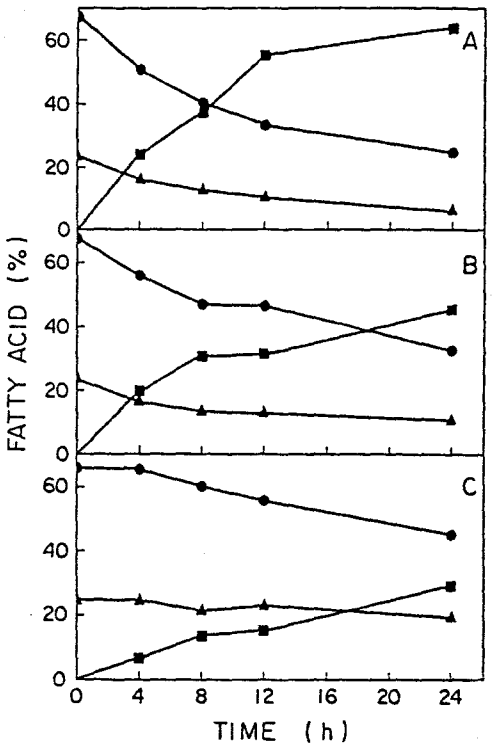

Fig. 1. Incorporation of *trans*-monoenoic fatty acids into cellular phospholipids. Cells (8.8×10^3/cm^2) were transferred to a dish with Eagle's medium supplemented with 6% delipidated fetal bovine serum, and further grown. After 24-h incubation, *trans*-9-18 : 1 (300 μM) (A), *trans*-11-20 : 1 (300 μM) (B), or *trans*-13-22 : 1 (400 μM) (C) was added to the medium. Phospholipids were isolated from cells, and their fatty acid compositions were determined as described in " MATERIALS AND METHODS." *cis*-monoenoic fatty acids (●); saturated fatty acids (▲); *trans*-monoenoic fatty acids (■).

and 30% of the total fatty acids of phospholipids, respectively. In the cells supplemented with trans-9-18:1, most of the trans-monoenoic fatty acids in phospholipids was trans-9-18:1. In the cells supplemented with trans-11-20:1, two thirds of the trans-monoenoic fatty acids in phospholipids was trans-11-20:1. About one third was trans-9-18:1. Upon the addition of trans-13-22:1 to the medium, trans-monoenoic fatty acids incorporated into phospholipids were

composed of trans-13-22:1 and trans-9-18:1. However,no incorporated trans-11-20:1 was detected.

Effect of trans-monoenoic fatty acids on phospholipid molecular species composition of V79-R cells

The composition of molecular species of phosphatidylcholine and phosphatidylethanolamine in the normal cells and in cells supplemented with trans-monoenoic fatty acids are shown in Table III. Novel phosphatidylcholine and phosphatidylethanolamine molecular species were formed. The molecular species of phosphatidylcholine and phosphatidylethanolamine were identified to be trans-9-18:1/cis-18:1 (21.0 and 29.2%), respectively, and trans-9-18:1/trans-9-18:1 (56.8 and 28.8%), respectively, in the cells supplemented with trans-9-18:1. When cells were grown in the presence of trans-11-20:1, the major molecular species of phosphatidylcholine and phosphatidylethanolamine containing trans-monoenoic fatty acids were trans-9-18:1/ cis-18:1, trans-11-20:1/cis-18:1, trans-11-20:1/ trans-9-18:1, and trans-11-20:1/trans-11-20:1. In the cells supplemented with trans-13-22:1, several molecular species were detected. Phosphatidylcholine and phosphatidylethanolamine contained 38.4 and 44.2% of molecular species containing trans-monoenoic fatty acids, respectively. The increase in the molecular species containing trans-monoenoic fatty acids resulted in a concomitant decrease of the major preexisting molecular species (16:0/cis-18:1, cis-18:1/cis-18:1).

TABLE III. Composition of molecular species of phospholipid in V79-R cells supplemented with *trans*-monoenoic fatty acids. Cells were grown in the presence of *trans*-monoenoic fatty acids as described in Table II. Molecular species of phospholipid were determined as described in " MATERIALS AND METHODS." Values represent the mean weigh % of total molecular species of two experiments. PC, phosphatidylcholine; PE, phosphatidylethanolamine

Molecular species	Addition							
	None		*Trans*-9-18 : 1		*Trans*-11-20 : 1		*Trans*-13-22 : 1	
	PC	PE	PC	PE	PC	PE	PC	PE
16 : 0/16 : 0	1.5	0.2	—	—	—	0.2	1.9	1.5
18 : 0/16 : 0	0.1	0.1	—	—	—	—	0.4	0.5
16 : 0/16 : 1	3.5	0.7	0.7	0.3	1.4	0.9	9.9	2.9
16 : 0/*cis*-18 : 1	33.7	23.1	3.0	3.5	2.5	7.9	25.3	12.3
18 : 0/*cis*-18 : 1	5.6	19.6	—	6.0	5.2	8.3	7.2	13.3
16 : 1/16 : 1	0.6	2.0	1.0	3.6	0.4	2.4	1.6	2.6
16 : 1/*cis*-18 : 1	11.3	9.4	0.7	3.5	—	4.4	5.2	6.4
cis-18 : 1/*cis*-18 : 1	40.0	39.4	2.6	7.6	5.2	11.7	7.7	16.4
trans-18 : 1/16 : 0	—	—	3.3	1.3	6.6	0.8	2.3	7.2
trans-18 : 1/18 : 0	—	—	0.6	1.2	—	—	—	6.1
trans-18 : 1/16 : 1	—	—	9.3	13.0	0.9	1.4	1.9	2.3
trans-18 : 1/*cis*-18 : 1	—	—	21.0	29.2	2.0	6.1	4.3	10.0
trans-18 : 1/*trans*-18 : 1	—	—	56.8	28.8	0.9	1.0	1.6	—
trans-20 : 1/16 : 0	—	—	—	—	2.3	4.6	—	—
trans-20 : 1/18 : 0	—	—	—	—	—	1.0	—	—
trans-20 : 1/16 : 1	—	—	—	—	8.0	1.9	—	—
trans-20 : 1/*cis*-18 : 1	—	—	—	—	23.0	19.4	—	—
trans-20 : 1/*trans*-18 : 1	—	—	—	—	15.2	6.1	—	—
trans-20 : 1/*trans*-20 : 1	—	—	—	—	20.3	18.1	—	—
trans-22 : 1/16 : 0	—	—	—	—	—	—	4.0	2.9
trans-22 : 1/18 : 0	—	—	—	—	—	—	0.5	1.3
trans-22 : 1/16 : 1	—	—	—	—	—	—	7.0	6.0
trans-22 : 1/*cis*-18 : 1 + *trans*-22 : 1/*trans*-18 : 1	—	—	—	—	—	—	14.2	7.6
trans-22 : 1/*trans*-22 : 1	—	—	—	—	—	—	0.9	—

Effect of trans-monoenoic fatty acids on activities of phospholipid synthesizing enzymes bound to membranes

Most of the enzymes catalyzing phospholipid synthesis in animal cells are associated with endoplasmic reticulum (86). Activities of some membrane-bound enzymes are known to be affected in response to changes in fatty acid composition of membrane phospholipids (69,91,92). Specific activities of phospholipid synthesizing enzymes in membrane fractions from the cells supplemented with trans-monoenoic fatty acids were

compared with those from the control cells (Table IV). No difference in the activities of cholinephosphotransferase and ethanolaminephosphotransferase, which catalyze the final step of the reactions forming phosphatidylcholine and phosphatidyl ethanolamine, respectively, were found between microsomal fractions from the control cells and the cells supplemented with trans-9-18:1. Trans-11-20:1 reduced the activities of both enzymes. The removal of 1,2-diolein from

TABLE IV. Phospholipid synthesizing enzyme activities from the cells grown with *trans*-monoenoic fatty acids. Conditions were described in "MATERIALS AND METHODS." Values are given as mean±S.E. ($n=5$). P values were calculated by Student's t-test.

Addition	Activity (nmol/min/mg protein)			
	Glycerophosphate acyltransferase	Cholinephospho-transferase	Ethanolaminephos-photransferase	Cholinephosphate cytidylyltransferase
None	6.42±0.36[a,b]	1.02±0.06[c]	0.79±0.01[d]	1.13±0.04
trans-9-18 : 1	15.08±0.29[a]	1.06±0.08	0.83±0.03	1.14±0.08
trans-11-20 : 1	3.20±0.15[b]	0.35±0.02[c]	0.06±0.002[d]	1.15±0.09

[a,b,c,d] $P<0.01$.

the reaction mixture had no effects on the activities of either enzyme (data not shown). This indicated that the addition of 1,2-diolein in the reaction mixture is not required, and that endogenous 1,2-diacylglycerol in microsomal membrane may be used as the substrate for the syntheses of phosphatidylcholine and phosphatidylethanolamine. The specific activity of glycerophosphate acyltransferase, which

catalyzes the reaction forming 1-acyl-sn-glycerol 3-phosphate, in the membrane fraction from the cells grown in the presence of trans-9-18:1 was increased to 2.3 times that of the control cells. When the cells were treated with trans-11-20:1, the enzyme activity in the membrane fraction was decreased to about 50% of that of the control cells. There was little difference in the activity of cholinephosphate cytidylyltransferase, which catalyzes the reaction forming CDP-choline, between the control cells and cells treated with trans-monoenoic fatty acids.

VI-4 DISCUSSION

Major molecular species of phospholipid in normal V79-R membranes are cis-18:1/cis-18:1 and 16:0/cis-18:1. In this chapter, I have shown that the addition of trans-monoenoic fatty acids to the medium resulted in the inhibition of V79-R cell growth, and that trans-monoenoic fatty acid and their metabolites were incorporated into phospholipids to form novel phospholipid molecular species containing trans-monoenoic fatty acids. The amount of phospholipids per cell increased when the cells were grown in the presence of trans-monoenoic fatty acids (Table II). The evidence is similar to that obtained with 16:0 (chapter IV) and cis-13-22:1 (chapter V). The increase in cellular phospholipid content may be due to the difference in the extent of inhibition by these fatty acids between cell division and phospholipid

synthesis.

On the other hand, two kinds of trans-monoenoic fatty acids had different effects on the activities of membrane-bound phospholipid synthesizing enzymes. The addition of trans-9-18:1 had no effect on the specific activities of cholinephosphotransferase, ethanolaminephosphotransferase, and cholinephosphate cytidylyltransferase, but stimulated the glycerophosphte acyltransferase activity. However, trans-11-20:1 decreased cholinephosphotransferase, ethanolamine-phosphotransferase, and glycerophosphate acyltransferase activities. Cholinephosphate cytidylyltransferase activity in microsome from the cells grown in the presence of trans-11-20:1 was similar to that from control cells.

Even though the rate of in vitro phospholipid synthesis was reduced by trans-11-20:1 (Table IV), the remaining activities may be enough to produce the amount of phospho-lipids necessary to the cells. After the addition of trans-monoenoic fatty acids, the amounts of phospholipids per cell increased, though cell division was inhibited. This sug-gests that the phospholipids gradually accumulated in the cells since the cells did not divide. Concomitant accumu-lation of phospholipids and inhibition of cell growth sug-gest that unusual phospholipid molecular species containing trans-monoenoic fatty acids and/or the decrease in the major preexisting phospholipid molecular species such as cis-18:1/cis-18:1 may be related to inhibition of cell growth and

division.

To explain the changes in the enzyme activities by trans-monoenoic fatty acids, the following possibilities are assumed. (a) Changes in physical properties of the membranes may alter the enzyme activities. Trans-9-18:1, trans-11-20:1, and trans-13-22:1 have higher melting points (44.5, 53.5, and 61.5°C) than that of cis-18:1 (13.4-16.3°C), which is the major fatty acid in the control cellular phospholipids. Our results show the incorporation of significant amounts of trans-monoenoic fatty acids into the cellular phospholipids. Generally, membrane functions are known to be affected by changes in membrane fluidity (5, 6). Decreased membrane fluidity caused by the production of new phospholipids molecular species containing trans-11-20:1 may have reduced the membrane enzyme activities. However, trans-9-18:1 did not reduce any enzyme activities. This may be due to little change in fluidity being caused by trans-9-18:1/trans-9-18:1 phosphatidylcholine (transition temp., 12°C (107)) at 37°C, which is a major phospholipid molecular species in the membranes of the cells treated with trans-9-18:1. (b) Phospholipid molecular species which are essential for the enzyme activities may be decreased by trans-monoenoic fatty acids. The incorporation of trans-monoenoic fatty acids into phospholipids caused a decrease in cis-18:1 / cis-18:1 phospholipid molecular species. There is a possibility that the cis-18:1/cis-18:1 molecular species is

required for the enzyme activities. The molecular species containing trans-9-18:1, such as trans-9-18:1/trans-9-18:1 and trans-9-18:1/ cis-18:1, may be able to substitute for the cis-18:1/cis-18:1 species for the enzyme activities. It is interesting that glycerophosphate acyltransferase activity may be stimulated by these molecular species. Previously we showed that cis-13-22:1 reduced glycerophosphate acyltransferase (chapter V). Since the melting point of cis-13-22:1 (34.7°C) is lower than that of trans-9-18:1 (44.5°C), the difference between the effects of cis-13-22:1 and trans-9-18:1 on glycerophosphate acyltransferase may not be caused by the fluidity of the molecular species, but by different structures of the molecular species. (c) We cannot exclude the possibility that there is a difference in availability of endogenous substrates for cholinephosphotransferase and ethanolaminephosphotransferase between trans-11-20:1-cells and control cells, since both enzymes utilized only endogenous diacylglycerol as substrates.

There is some evidence that cholinephosphate cytidylyltransferase is activated upon translocation from the cytoplasm to the endoplasmic reticulum to regulate the biosynthesis of phosphatidylcholine (106, 108-110). Exogenous trans-monoenoic fatty acids did not enhance membrane bound cholinephosphate cytidylyltransferase activity, in contrast to the effects of oleic and palmitic acids as described by Pelech et al. (110).

VII SUMMARY

CHAPTER II

When lipids were removed from the culture medium, growth of V79 cells ceased. Supplementation with cis-octadecenoic acids satisfied the requirement for lipids by V79 cells. After starvation of the exogenous lipids by the shift-down of the medium to lipid-free medium, the content of octadecenoic acid in phospholipids increased more slowly in V79 cells than in V79-R cells, which can grow in the lipid-starved medium. The incorporation of [^{14}C] acetic acid into monoenoic fatty acids and phospholipid molecular species containing monoenoic fatty acids in V79 cells was lower than that in V79-R cells. The reduced formation of monoenoic fatty acids was shown to be due to deficiency in the stimulation of activity of stearoyl-CoA desaturase which is a key enzyme to convert saturated fatty acids to monoenoic fatty acids.

CHAPTER III

V79-R Cells grown in lipid-free medium contained octadecenoic acids as the major fatty acids esterified to lipids. Octadecenoic acids were composed of two positional isomers, oleic and cis-vaccenic acids. The distribution of oleic and cis-vaccenic acids was altered by the addition of various fatty acids to the medium. There was no difference

in the distribution of oleic and cis-vaccenic acids in phospholipids between mitochondria and microsomes. Cardiolipin contained higher amounts of palmitoleic and cis-vaccenic acids than did other lipids.

CHAPTER IV

The addition of palmitic acid or myristic acid to a medium containing delipidated fetal bovine serum resulted in severe inhibition of V79-R cell growth. The degree of inhibition by palmitic acid was concentration dependent. Simultaneous addition of oleic acid protected the cells from the inhibition by palmitic acid. In the presence of palmitic acid , total phospholipid and triacylglycerol per cell increased to two- and 13-fold, respectively. Palmitic acid caused an increase in the proportion of palmitic acid with concomitant decrease of oleic acid in phosphatidylcholine , phosphatidylethanolamine and triacylglycerol. Palmitic acid inhibited the synthesis of phospholipid molecular species with two monoenoic fatty acids. However, membrane flow of phospholipids from endoplasmic reticulum to plasma membrane was preserved. About 70% of the triacylglycerol molecular species were those containing three saturated or two saturated and one monoenoic fatty acids. Electron microscopy revealed a large amount of triacylglycerol and fiber-like membrane structures in the cells supplemented with palmitic acid.

CHAPTER V

The relationship between the inhibition of cell growth and the changes in phospholipid metabolism in the presence of erucic acid was studied in chinese hamster V79-R cells. The addition of erucic acid to the medium inhibited cell growth. The degree of inhibition by erucic acid at a given concentration was dependent on cell density. Exogenous erucic acid was incorporated into cellular phospholipids to form new phospholipid molecular species, which were identified to be the erucoyl/oleoyl, erucoyl/gondoyl and erucoyl/erucoyl species. Synthesis of phosphatidylcholine and phosphatidylethanolamine in endoplasmic reticulum was reduced by erucic acid. Erucic acid had no effect on membrane flow of phospholipids from endoplasmic reticulum to plasma membrane. The specific activity of sn-glycerol 3-phosphate acyltransferase in the membrane fraction from the cells supplemented with erucic acid was lower than that from the control cells. The reduction of phospholipid synthesis was attributed to the decrease in sn-glycerol 3-phosphate acyltransferase activity.

CHAPTER VI

The interrelationship between the inhibition of cell growth and changes in phospholipid molecular species was studied in the presence of elaidic, trans-11-eicosenoic, or brassidic acids in chinese hamster V79-R cells. The ad-

dition of trans-monoenoic fatty acids to the medium inhibited cell growth and caused an increase in the total cellular content of phospholipids. However, there was no difference in the polar head group composition of these phospholipids among all the cells supplemented with trans-monoenoic fatty acids. Exogenous trans-monoenoic fatty acids were incorporated into cellular phospholipids to form novel phospholipid molecular species.

Phospholipid synthesizing enzyme activities bound to the membranes composed of phospholipid molecular species of trans-monoenoic fatty acids were determined. Cholinephospho transferase [EC 2.7.8.2] and ethanolaminephosphotransferase [EC 2.7.8.1] activities were decreased by trans-11-eicosenoic acid, but not changed by elaidic acid. Glycerophosphate acyltransferase [EC 2.3.1.15] activity was increased by elaidic acid and decreased by trans-11-eicosenoic acid. Cholinephosphate cytidylyltransferase [EC 2.7.7.15] activity was not changed by trans-monoenoic fatty acids.

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