Coenzyme A-dependent modification of fatty acyl chains of rat liver membrane phospholipids: possible involvement of ATP-independent acyl-CoA synthesis

Takayuki Sugiura,[1] **Naomi Kudo, Takayuki Ojima, Sachiko Kondo, Atsushi Yamashita, and Keizo Waku**

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan

Abstract Certain species of fatty acyl chains of microsomal lipids from rat hepatocytes underwent desaturation when the microsomal fraction was incubated with CoA and NADH. For instance, 18:0, 18:2(n-6), and 20:3(n-6) incorporated into membrane lipids were gradually converted to 18:1(n-9), 18:3(n-6), and 20:4(n-6), respectively. Further, 20:5(n-3) and 18:3(n-6) were metabolized to 22:5(n-3) and 20:4(n-6), respectively, through chain elongation or chain elongation plus subsequent desaturation when malonyl-CoA was present. In contrast to esterified fatty acids, negligible change was observed for free fatty acids under the same experimental conditions. It is apparent that ATP-independent acyl-CoA synthesis is implicated in such enzymatic modification of fatty acyl chains. The presence of either gel-filtered cytosol or bovine serum albumin markedly potentiated the reaction. However, the addition of ATP and Mg^{2+} did not accelerate the overall reaction induced in the presence of CoA alone. These results suggest that the pathway of CoA-dependent/ATP-independent modification of fatty acyl chains is sufficiently active to account for the desaturation and chain elongation of fatty acids esterified in membrane lipids, especially phospholipids, of living cells.—**Sugiura, T., N. Kudo, T. Ojima, S. Kondo, A. Yamashita, and K. Waku.** Coenzyme A-dependent modification of fatty acyl chains of rat liver membrane phospholipids: possible involvement of ATP-independent acyl-CoA synthesis. *J. Lipid Res.* 1995. **36:** 440–450.

Supplementary key words desaturation • chain elongation • fatty acid metabolism • liver microsomes • hepatocytes

Mammalian tissues contain various types of fatty acids with different chain lengths and with different degrees of unsaturation as constituents of various lipid molecules (1). These fatty acids are essential to mammalian tissues, first of all, as the hydrophobic moieties of the lipid bilayer of various biomembranes and also as a source of energy. In addition to these structural and nutritional roles, certain types of polyunsaturated fatty acids esterified in phospholipids, especially arachidonic acid (20:4), have another important role as precursors of potent lipid mediators such as prostaglandins and leukotrienes (2). Several tissues are known to accumulate particular species of fatty acids in lipid molecules, such as C22 polyunsaturated fatty acids in the retina and testis (3); these fatty acids are also assumed to play physiologically important roles in these tissues, though their biological functions have not yet been fully elucidated.

It is well known that various mammalian tissues can synthesize several types of fatty acids such as 16:0 via the de novo synthetic pathway. A considerable part of the fatty acids present in mammalian tissues, especially polyunsaturated fatty acids, is, however, usually supplied through the diet. Fatty acids derived from the diet are delivered through the lymph and blood stream to peripheral tissues in the form of various lipid molecules. A portion of fatty acids either synthesized de novo or derived from the diet is known to be further modified in the liver and other peripheral tissues through the chain elongation and/or desaturation reactions. Such modifications are important in providing a variety of appropriate fatty acids to individual tissues.

There have been numerous studies on the mechanisms of chain elongation and desaturation of fatty acids in mammalian tissues and cells (4–15). It has been widely accepted that acyl-CoAs are substrates for chain elongation and desaturation of fatty acids in mammalian tissues. Hence, most studies on the enzymatic modifications of fatty acids have been carried out using acyl-CoA as the

Abbreviations: BSA, bovine serum albumin; MEM, minimum essential medium; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography. Fatty acids are designated in terms of number of carbon atoms:number of double bonds, e.g., 18:1 for oleic acid.

[1]To whom correspondence should be addressed.

substrate. Free fatty acids themselves do not act as the substrate for either chain elongation or desaturation and require conversion to fatty acyl-CoA prior to enzymatic modifications. Moreover, in the case of fatty acids esterified in various lipid molecules, it has generally been assumed that fatty acids are liberated from the parent lipid molecules by (phospho)lipases and then converted to acyl-CoA by acyl-CoA synthetase with the consumption of ATP in order to undergo subsequent enzymatic modifications.

Recently, however, Sugiura, Masuzawa, and Waku (16) and Stymne and Stobart (17) showed that considerable amounts of acyl-CoA are formed when microsomes are incubated with CoA alone in the absence of ATP. Interestingly, among the acyl-CoA species formed through the reaction of ATP-independent acyl-CoA synthesis, 18:0-CoA is a good substrate for Δ9 desaturase and 18:2-CoA is a preferred substrate for Δ6 desaturase (10, 13, 15). The possibility, therefore, arises that a portion of the acyl-CoA formed through the reaction of ATP-independent acyl-CoA synthesis is enzymatically modified before being returned to the parent phospholipid molecules or other lysophospholipids, because Δ9 desaturation and Δ6 desaturation as well as chain elongation are very common reactions in various mammalian tissues.

In the present study, we examined this possibility in detail. We found that desaturation or chain elongation of fatty acids esterified in microsomal lipids actually takes place in the presence of CoA and NADH or NADH plus malonyl-CoA even in the absence of ATP and Mg^{2+}. Several lines of evidence presented here strongly suggest that ATP-independent transient acyl-CoA synthesis is of physiological importance in the modifications of certain types of fatty acyl chains of membrane phospholipids.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade. [1-^{14}C]18:0 (58.0 mCi/mmol), [1-^{14}C]18:2(n-6) (50.0 mCi/mmol), [1-^{14}C]20:3(n-6) (47.0 mCi/mmol), [1-^{14}C]20:5(n-3) (52.0 mCi/mmol) and [5,6,8,9,11,12,14,15,17,18-^{3}H]20:5(n-3) (116.8 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, MA). [^{3}H]20:5 was diluted with unlabeled 20:5 to 60 mCi/mmol. Collagenase and aprotinin were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Collagen-coated plastic dishes were from Iwaki Glass (Tokyo, Japan). Soybean trypsin inhibitor, bovine serum albumin (BSA), apyrase, dexamethasone, and malonyl-CoA were from Sigma (St. Louis, MO). Unlabeled fatty acids were obtained from Sigma (St. Louis, MO), Nu-Chek Prep (Elysian, MN), and Matreya, Inc. (Pleasant Gap, PA). Eagle's minimal essential medium (MEM) containing Kanamycin was obtained from Nissui (Tokyo, Japan). McCoy's 5A medium was from Gibco Laboratories (New York, NY). Insulin (porcine) was obtained from Biomedical Technologies Inc. (Stoughton, MA). Kanamycin was from Meiji Seika Co. Ltd. (Tokyo, Japan). CoA was from Kyowa Hakko Co. Ltd. (Tokyo, Japan). Ampure™SA was purchased from Amersham (Amersham, UK).

Animals

Wistar male rats (body weight, 200–250 g) were obtained from Sankyo Labo Service (Tokyo, Japan) and were fed laboratory chow (Clea Japan, Tokyo, Japan) and water ad libitum. Rats were fasted for 24 h prior to the experiments. In the experiments where the enzyme activities of the microsomal fraction were estimated, fasted (48 h)-refed (with a fat-free diet for 16 h) rats were used.

Preparation of hepatocytes

Hepatocytes were prepared from rat livers by in situ perfusion with collagenase according to the method of Seglen (18). Briefly, rats were first anesthetized with pentobarbital and their livers were perfused in situ with 100 ml 10 mM HEPES-buffered isotonic solution (pH 7.2) containing 0.5 mM EGTA for 5 min. The livers were then perfused with 100 ml 10 mM HEPES-buffered collagenase solution (pH 7.5) for 5–10 min. After the perfusion, the livers were excised, placed in dishes containing MEM (pH 7.3), and minced with a scalpel. Hepatocytes were dispersed into the medium by gentle shaking and pipetting. The hepatocyte suspension was filtered once through a stainless steel mesh to remove connective tissues and cell clumps and centrifuged at 50 *g* for 1 min. The sedimented cells were washed 4 times and resuspended in MEM.

Incorporation and metabolism of radiolabeled fatty acids in hepatocytes

Hepatocytes prepared under sterile conditions were suspended in 5 ml HEPES-MEM at a density of 2.5 × 10^5 cells/ml and seeded in plastic dishes (21 cm^2 or 55 cm^2, 1.25 × 10^6 or 2.5 × 10^6 cells/dish, respectively) coated with collagen. Cells were incubated in a CO_2 incubator (5% CO_2, 95% air) at 37°C for 4 h. Then the medium was removed and replaced with McCoy's 5A medium containing insulin (10^{-8} M), dexamethasone (10^{-9} M), aprotinin (5 KIU/ml), and Kanamycin (30 mg/l). Cells were further incubated for 48 h in the medium. The medium was removed and replaced with the same medium containing 0.1% BSA and radiolabeled fatty acids (0.02 μCi/ml). Cells were incubated with radiolabeled fatty acids for 1 h. The medium was then aspirated and the cells were washed with MEM. The washed cells were incubated in McCoy's 5A medium containing insulin, dexamethasone, and aprotinin for a further 1–6 h. At the end of the respective incubation periods, the medium was removed, the

dishes were washed with 5 ml 10 mM phosphate-buffered saline (pH 7.3) and the cells were collected with a rubber policeman. The incubation was terminated by the addition of chloroform–methanol 1:2 (v/v). Total lipids were extracted according to the method of Bligh and Dyer (19). Fatty acyl residues (esterified and nonesterified) were then converted to fatty acid methyl esters by treatment with 5% HCl-methanol at 70°C for 2 h (20). The resultant fatty acid methyl esters were dissolved in acetonitrile after the removal of methanolic HCl under a stream of N_2 gas. The sample was injected into an HPLC system (Shimadzu, LC6A, Kyoto, Japan) equipped with a reverse-phase column (LiChrosorb RP-18, Merck, Darmstadt, Germany) (21). Acetonitrile–water 9:1 was used to separate individual fatty acid methyl esters according to the method of Aveldano, Van Rollins, and Horrocks (22). Each fraction was collected in a counting vial, and the radioactivity was estimated in a liquid scintillation counter (Aloka, LSC-3500, Tokyo, Japan). The rates of conversion of fatty acids were calculated from the radioactivity in each fraction.

Preparation of microsomal fraction of hepatocytes prelabeled with radioactive fatty acids

Isolated hepatocytes (5×10^8 cells/60 ml) from starved-refed rats were incubated with radiolabeled fatty acids (50 μCi/60 ml) in HEPES-MEM containing 0.05% BSA at 37°C for 1 h. The cells were spun down at 180 *g* for 15 min at 4°C and further washed twice with HEPES-MEM containing 0.05% BSA. Sedimented cells were homogenized in 20 mM Tris-HCl buffer containing 2 mM EDTA (pH 7.3) with a Dounce homogenizer. An equal volume of 20 mM Tris-HCl-buffered 0.5 M sucrose containing 2 mM EDTA was then added to the homogenate, and the mixture was centrifuged at 11000 *g* for 15 min. The supernatant was centrifuged again at 11000 *g* for 15 min, and the resulting supernatant obtained was ultracentrifuged at 105000 *g* for 1 h. The pellet was washed once with 20 mM Tris-HCl-buffered 0.25 M sucrose (pH 7.3) containing 2 mM EDTA and ultracentrifuged at 105000 *g* for 1 h. The obtained microsomal fraction was immediately used for enzyme reactions. The protein contents were measured according to the method of Lowry et al. (23).

ATP-independent synthesis of acyl-CoA from endogenous membrane lipids

Freshly prepared rat liver microsomes (5 mg protein) and CoA (final 10 μM–1 mM) were incubated in 5 ml 10 mM HEPES buffer (pH 7.3) containing 1 mM EDTA and 50 mg BSA (final 1%, w/v) at 37°C for 30 min. The mixture without microsomes was incubated in the same manner as a blank. All incubations were carried out in triplicate. The enzyme reaction was stopped by adding 18.8 ml chloroform–methanol 1:2 (v/v) and then 25 nmol 17:0-CoA was added as an internal standard. After the addition of 6.3 ml chloroform and 6.3 ml water according to the method of Bligh and Dyer (19), the mixture was shaken vigorously and then centrifuged. The water–methanol layer was carefully transferred into another tube containing 12.5 ml chloroform. The tube was shaken vigorously and centrifuged. These procedures were repeated to remove chloroform-soluble material thoroughly. After washing with chloroform, the water–methanol layer was evaporated under a stream of N_2 gas and then under vacuum. Ethanol was added repeatedly to remove water. The residue was dissolved in 0.5 ml ethanol–water 4:1 (v/v) and applied to TLC plates (20 cm wide) that had been prewashed with methanol and dried before use. The plates were developed with n-butanol–acetic acid–water 5:2:3 (v/v). After development, the plates were dried and sprayed with primuline to visualize the band of acyl-CoA under ultraviolet light. The band corresponding to that of standard acyl-CoA was scraped off into a tube. The acyl moiety of acyl-CoA was transmethylated using 0.5 M methanolic sodium methoxide with stirring. The resultant fatty acid methyl esters were extracted with hexane and analyzed by GLC using a gas chromatograph (GC-8A, Shimadzu, Kyoto, Japan) equipped with a capillary column fused with SP2330 (Supelco, Bellefonte, PA). Samples of fatty acid methyl esters were prepared no longer than 24 h after the preparation of microsomes.

Desaturation and chain elongation of fatty acyl residues of microsomal lipids

Desaturation of fatty acids was explored using the incubation mixture described by Mahfouz, Valicenti, and Holman (24) with slight modifications, such as the use of the incubation mixture without ATP and $MgCl_2$. Briefly, a microsomal fraction (0.5 mg protein) obtained from hepatocytes prelabeled with ^{14}C-labeled or ^{3}H-labeled fatty acids was incubated in 0.5 ml of 50 mM Tris-HCl buffer (pH 7.3) containing 0.15 M KCl, 0.25 M sucrose, 1 mM NADH, 1.5 mM glutathione, 45 mM NaF, 0.5 mM nicotinamide, and 1 mM EDTA in the presence or absence of 100 μM CoA. EDTA was replaced with 5 mM ATP and 5 mM $MgCl_2$ in some tubes. The incubation was performed at 37°C for 30–180 min. In some experiments, BSA (0.1–0.25%) or rat liver cytosol (0.5 mg protein) that had been dialyzed or gel-filtered through a small column of Ampure™SA to remove low molecular weight materials (<5000 daltons) prior to use was added to the incubation mixtures. In order to examine chain elongation, malonyl-CoA (100 μM) was added in addition to CoA, and NADPH (1 mM) was added together with NADH. All incubations were stopped by adding chloroform–methanol 1:2 and total lipids were extracted by the method of Bligh and Dyer (19). Fatty acyl residues were converted to fatty acid methyl esters using 5% HCl–methanol and were analyzed by HPLC. In some experi-

ments, the enzyme reaction was stopped by adding 1 ml of 10% KOH-methanol. The saponification was performed at 85°C for 40 min. The resultant fatty acids were extracted, after the addition of 2 ml 4 N HCl, with petroleum ether. Fatty acids were methylated with 5% HCl-methanol as described above. Similar experimental results were obtained by these two methods. In another set of experiments, total lipids were fractionated on TLC plates developed with petroleum ether-diethyl ether-acetic acid 70:30:1 (v/v). The individual lipids were extracted by the method of Bligh and Dyer (19) and the fatty acyl residues were converted to fatty acid methyl esters and analyzed by HPLC.

Desaturation of free fatty acid by microsomal fraction

A rat liver microsomal fraction was obtained from starved-refed rats in the same way as described for isolated hepatocytes. The microsomal fraction (0.5 mg protein) was incubated in 0.5 ml of 50 mM Tris-HCl buffer (pH 7.3) containing 100 μM [^{14}C]18:2 (140,000 dpm), 0.25% BSA, 1 mM EDTA, and other materials as described above in the presence or absence of 100 μM CoA. In some tubes, EDTA was omitted and 5 mM ATP and 5 mM $MgCl_2$ were included. Gel-filtered cytosol (0.5 mg protein) was also added to some tubes. The enzyme reaction was carried out at 37°C for 30 min and terminated by adding 1 ml 10% KOH-methanol and heating (85°C for 40 min). Fatty acids were extracted and converted to fatty acid methyl esters prior to HPLC analysis as described above.

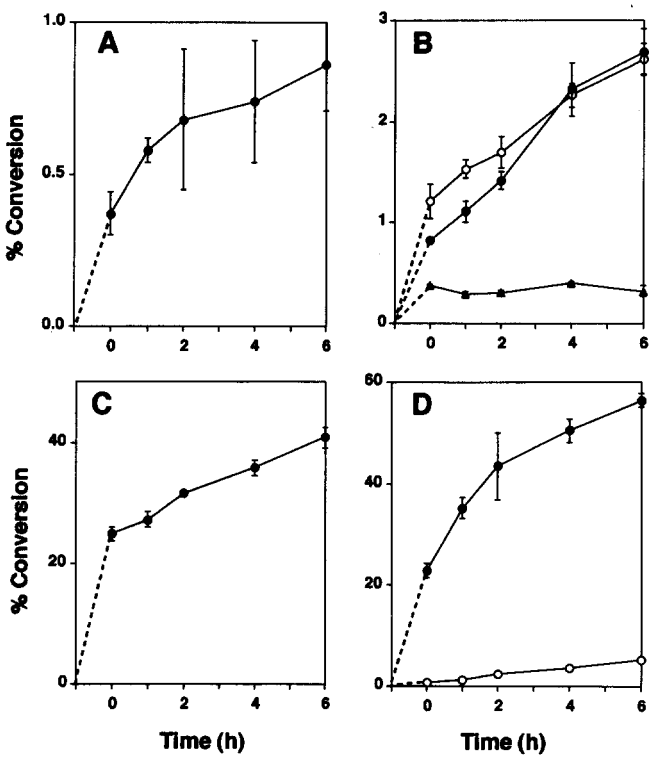

Fig. 1. Metabolic conversion of radiolabeled fatty acids in hepatocytes. Rat hepatocytes prelabeled (for 60 min) with radiolabeled fatty acids were further incubated for 1-6 h. At the end of the respective incubation periods, the cells were collected and total lipids were extracted. Total fatty acids were then methylated and the resultant fatty acid methyl esters were analyzed by HPLC as described in Materials and Methods. The rates of conversion of fatty acids were calculated from the radioactivities in each fraction. (A), [^{14}C]18:0→18:1(n-9); (B), [^{14}C]18:2(n-6)→18:3(n-6) (▲), 20:3(n-6) (○), 20:4(n-6) (●); (C), [^{14}C]20:3(n-6)→20:4(n-6); (D), [^{14}C]20:5(n-3)→22:5(n-3) (●), 22:6(n-3) (○). The data are the means ± SD from three determinations.

Separation of individual lipid classes

Total lipids were fractionated on TLC plates developed with petroleum ether-diethyl ether-acetic acid 70:30:1 (v/v). The R_f values for cholesteryl esters, triacylglycerols, free fatty acids, 1,3-diacylglycerols, 1,2-diacylglycerols, monoacylglycerols, and phospholipids were 0.98, 0.79, 0.37, 0.22, 0.19, 0.02, and 0.00 (the origin), respectively. In some experiments, individual phospholipids were separated by two-dimensional TLC developed first with chloroform-methanol-28% NH_4OH 65:35:5 (v/v) and then with chloroform-acetone-methanol-acetic acid-water 5:2:1:1.3:0.5 (v/v) (20). Lipid spots were detected under UV light after spraying the plates with 0.001% primuline in acetone-water 4:1 (v/v). Each spot was scraped into a counting vial and the radioactivity was estimated. In some experiments, lipids were extracted from the silica gel by the method of Bligh and Dyer (19).

Statistical analysis

Student's *t*-test was used for statistical analysis.

RESULTS

Incorporation and metabolism of radiolabeled fatty acids in intact hepatocytes

First, the incorporation and the metabolism of various radiolabeled fatty acids in cultured rat hepatocytes were studied. Cells were incubated with radiolabeled fatty acids (0.1 μCi/dish) for 1 h and then washed with MEM. During the above incubation period, 12% ([^{14}C]18:0), 27% ([^{14}C]18:2(n-6)), 14% ([^{14}C]20:3(n-6)) and 24% ([^{14}C]20:5(n-3)) of the radiolabeled fatty acids among individual lipid classes were taken up by cells. The incorporated radiolabeled fatty acids other than 18:0 were located mainly in the triacylglycerol (60-70%) and phospholipid (20-40%) fractions in each case, and the proportions of the radioactivities found in the free fatty acid fraction were very low (1-2%), although a large part of the radioactivity (80%) was still detected in the free fatty acid fraction in the case of 18:0. The distribution of the radioactivity of labeled fatty acids among individual lipid classes did not change dramatically during the prolonged incubation after a wash (data not shown).

We further examined whether these radiolabeled fatty acids are metabolized through desaturation and/or chain elongation reaction after being incorporated into cells. **Figure 1** shows the metabolic conversion of radiolabeled

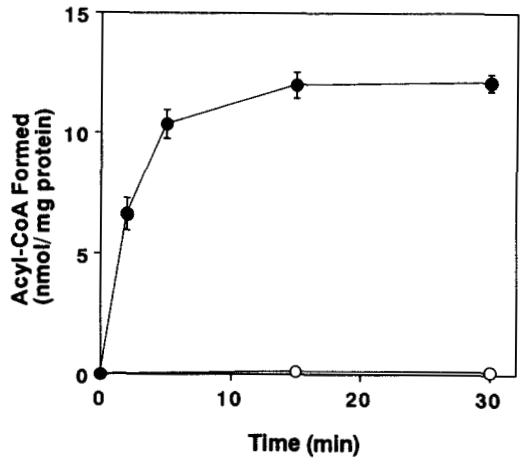

Fig. 2. Formation of acyl-CoA from free fatty acids, CoA and ATP-Mg^{2+}. Rat liver microsomes (1 mg/ml) were incubated with free [^{14}C]18:2 (final 100 μM) and CoA (final 100 μM) in 20 mM HEPES buffer (pH 7.3) containing BSA (1%) in the presence of ATP (5 mM) and $MgCl_2$ (5 mM) (●) or in the presence of EDTA (1 mM) (○) at 37°C for indicated periods. The enzyme reaction was stopped by the addition of chloroform–methanol according to the method of Bligh and Dyer (19). The radioactivity recovered from the water–methanol layer was estimated. The data are the means ± SD from four determinations.

fatty acids in cultured rat hepatocytes. 18:0, 18:2(n-6), 20:3(n-6), and 20:5(n-3) were gradually metabolized to 18:1(n-9), 20:4(n-6) plus 20:3(n-6), 20:4(n-6), and 22:5(n-3) plus 22:6(n-3), respectively, during the prolonged incubation of cells after the removal of unincorporated fatty acids. The magnitude of the modification of individual fatty acids was, however, different among fatty acid species. The metabolic conversion of 20:3(n-6) and 20:5(n-3) was pronounced compared with that of 18:0 and 18:2(n-6) as was the case for the metabolic conversion observed before the removal of unincorporated fatty acids. We also confirmed that 20:4(n-6) formed from 18:2(n-6) accumulated mainly in the phospholipid fraction (1650 dpm/2.5 × 10^6 cells) rather than in the triacylglycerol fraction (160 dpm/2.5 × 10^6 cells) during 6 h incubation, although a small increase of 20:3(n-6) was observed in the triacylglycerol fraction (530 dpm/2.5 × 10^6 cells) in addition to in the phospholipid fraction (670 dpm/2.5 × 10^6 cells).

ATP-independent acyl-CoA synthesis from rat liver microsomal lipids

We then explored the possibility that ATP-independent acyl-CoA synthesis from membrane phospholipids is implicated in the metabolic conversion of fatty acids incorporated into cellular lipids as described above. Prior to studying ATP-independent acyl-CoA synthesis, we examined the conditions for the formation of acyl-CoA from free fatty acid using radiolabeled fatty acids as substrates. As shown in **Fig. 2,** 18:2-CoA was synthesized only when exogenous CoA, ATP, and Mg^{2+} were present. We could not detect the formation of 18:2-CoA when ATP and Mg^{2+} were omitted from the incubation mixture. It is clear, therefore, that the presence of CoA alone is not sufficient for the synthesis of acyl-CoA from free fatty acid, at least under the present experimental conditions.

However, we confirmed that acyl-CoA was formed dose-dependently and time-dependently when microsomes and CoA without added fatty acids were incubated even in the absence of exogenous ATP and Mg^{2+}. **Table 1** shows the fatty acid profiles of the newly formed acyl-CoA. We found that there are rather strict selectivities of the fatty acyl moiety in the formation of acyl-CoA. 20:4-CoA, 18:0-CoA, and 18:2-CoA were preferentially synthesized from endogenous lipids by the addition of CoA. In contrast, the rates of the formation of 18:1-CoA and 22:6-CoA were very small (<4 nmol/30 min per 5 mg protein). It was calculated that about 1/140 of the total fatty acyl moieties of the membrane lipids was converted to acyl-CoA in the presence of 100 μM CoA. Considering that 18:0, 18:2, and 20:4 are mainly esterified in the phospholipid fraction in rat liver microsomes (96%, 86%, and 97%, respectively), it appears that the origin of the acyl moiety of the generated acyl-CoA is phospholipids rather than neutral lipids.

Desaturation and chain elongation of fatty acyl moieties of membrane lipids in microsomal fraction obtained from hepatocytes

Figure 3 shows the effects of CoA on the desaturation or chain elongation of fatty acyl moieties of membrane lipids in the microsomal fraction obtained from hepato-

TABLE 1. Fatty acyl moieties of acyl-CoA formed during the incubation of liver microsomes with CoA

Addition	16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:4	22:5	22:6	Total
					nmol/30 min/5 mg protein						
Without CoA	1.7 ± 0.3	0.1 ± 0.1	0 ± 0	0.2 ± 0.2	0.5 ± 0.9	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.3 ± 0.3	2.8
With 10 μM CoA	2.3 ± 0.4	0.2 ± 0.1	0.5 ± 0.4	0.2 ± 0.3	0.4 ± 0.1	0 ± 0	3.3 ± 1.0	0 ± 0	0 ± 0	0.3 ± 0.5	7.2
With 100 μM CoA	1.7 ± 0.4	0.2 ± 0.2	2.8 ± 0.5	1.0 ± 0.5	3.3 ± 0.5	0 ± 0	24.9 ± 1.2	0 ± 0	0 ± 0	1.0 ± 0.3	34.9
With 1 mM CoA	6.7 ± 0.4	0.7 ± 1.1	21.6 ± 2.0	3.3 ± 1.1	16.5 ± 0.8	1.6 ± 0	78.9 ± 11.9	0 ± 0	0.1 ± 0.2	0.3 ± 0.3	129.7

Rat liver microsomes were incubated with or without CoA (final 10 μM–1 mM) in 10 mM HEPES buffer (pH 7.3) containing EDTA (1 mM) and BSA (1%) at 37°C for 30 min. The reaction was stopped by adding chloroform–methanol 1:2. After the addition of 17:0-CoA (25 nmol) as an internal standard, acyl-CoA was extracted and purified as described in Experimental Procedures. Fatty acyl moieties of acyl-CoA were converted to fatty acid methyl esters using 5% HCl–methanol and analyzed by GLC. TLC plates without samples were run as blanks. The data are means ± SD from three determinations.

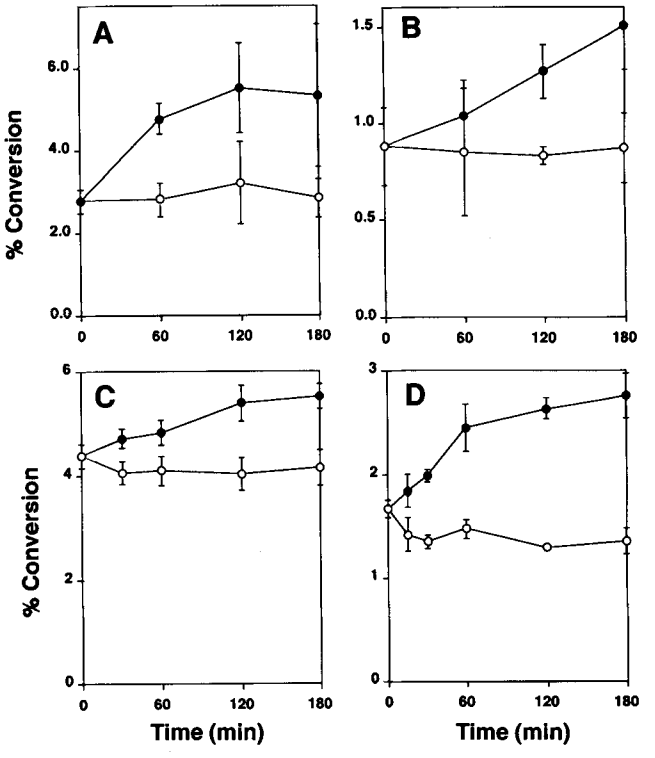

Fig. 3. CoA-dependent metabolic conversion of fatty acyl chains of microsomal lipids. Rat hepatocytes were prelabeled with ^{14}C- or ^{3}H-labeled fatty acids for 60 min. The cells were washed, homogenized, and centrifuged to obtain the microsomal fraction. Washed microsomal fractions (0.5 mg protein) were incubated with NADH (1 mM) in 0.5 ml of 50 mM Tris-HCl buffer (pH 7.3) containing several materials in the presence or absence of CoA (100 μM) at 37°C as described in Experimental Procedures. In the case of [^{3}H]20:5(n-3), NADPH (1 mM) was added together with NADH, and malonyl-CoA (100 μM) was added in addition to CoA (100 μM). Total fatty acyl chains were converted to fatty acid methyl esters and analyzed by HPLC as described in Materials and Methods. (A), [^{14}C]18:0→18:1(n-9); (B), [^{14}C]18:2(n-6)→18:3(n-6); (C), [^{14}C]20:3(n-6)→20:4(n-6); (D), [^{3}H]20:5(n-3)→22:5(n-3). (●) with CoA; (○) without CoA. For panel (D), (●) with CoA and malonyl-CoA; (○) without CoA and malonyl-CoA. The data are the means ± SD from three to four determinations.

cytes prelabeled with radiolabeled fatty acids. In the absence of CoA, neither desaturation nor chain elongation was observed, even in the presence of NADH (or NADH plus NADPH). In contrast, enzymatic conversion proceeded when CoA was included in the incubation mixture. 18:0 was metabolized by Δ9 desaturase to 18:1(n-9), 18:2(n-6) was metabolized by Δ6 desaturase to 18:3(n-6), and 20:3(n-6) was metabolized by Δ5 desaturase to 20:4(n-6). 20:5(n-6) was metabolized by chain elongation reaction to 22:5(n-3) when malonyl-CoA was added together with CoA.

The effects of cytosol and BSA on CoA-dependent modification of the fatty acyl moieties of microsomal lipids were next examined. **Figure 4** illustrates the case of [^{14}C]18:2(n-6). Desaturation did not take place when CoA was omitted from the incubation mixture. The desaturation of 18:2(n-6) to 18:3(n-6) occurred only when CoA was present. On the other hand, the addition of cytosol, dialyzed cytosol, or gel-filtered cytosol enhanced the desaturation reaction. The effect of cytosol was abolished (to the basal level) when boiled cytosol was used (data not shown), suggesting that cytosolic proteins are involved in the potentiation. The presence of BSA also potentiated the reaction to some extent. Therefore, we added BSA or gel-filtered cytosol to the reaction mixture in the following experiments.

We then checked the effect of the addition of malonyl-CoA on CoA-dependent modification of the fatty acyl chains of membrane lipids. In the presence of malonyl-CoA in addition to CoA, 18:2(n-6) was effectively converted to 20:4(n-6) and 20:3(n-6) with a concomitant decrease in the formation of 18:3(n-6) (data not shown); this is in accord with the metabolic conversion of 18:2(n-6) to 20:4(n-6) and 20:3(n-6) observed in intact cells as described previously.

Next we examined in which lipid fraction the desaturation of 18:2(n-6) to 18:3(n-6) takes place. As shown in **Figure 5,** 18:3(n-6) formed from 18:2(n-6) is present in both the phospholipid and triacylglycerol fractions, although the magnitude of the desaturation observed during the incubation with CoA was somewhat pronounced in the phospholipid fraction compared with triacylglycerol fraction (2-fold). We confirmed that a portion of the desaturated fatty acids is actually present in the phosphatidylcholine fraction which accounts for 85% of the total radioactivity in the phospholipid fraction.

Because a small amount of free fatty acid (8.9 ± 5.7% of the total radioactivity, mean ± SD of 11 separate experiments) was present in the microsomal fraction used here, we checked whether free fatty acids are involved in

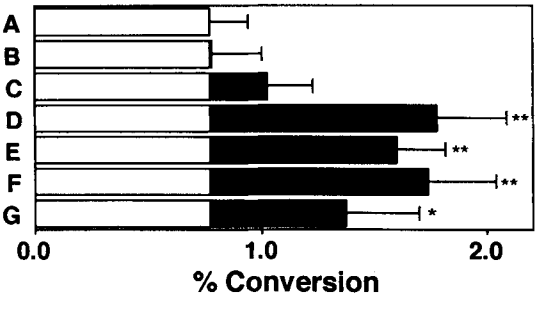

Fig. 4. The effects of cytosol and BSA on CoA-dependent metabolic conversion of [^{14}C]18:2(n-6) incorporated into microsomal lipids. Hepatocytes were prelabeled with [^{14}C]18:2(n-6) for 60 min. Cells were washed, homogenized, and centrifuged to obtain the microsomal fraction. The washed microsomal fraction (0.5 mg protein) was incubated with NADH (1 mM) and CoA (100 μM) in the presence or absence of cytosol or BSA. The incubation was carried out at 37°C for 120 min. The rates of conversion of [^{14}C]18:2(n-6) to 18:3(n-6) were determined. (A), before incubation; (B), without CoA; (C), with CoA; (D), with CoA and cytosol (0.5 mg protein); (E), with CoA and dialyzed cytosol (0.5 mg protein); (F) with CoA and gel-filtered cytosol (0.5 mg protein); (G) with CoA and BSA (0.5 mg). The solid bars represent the rates of conversion during the enzyme reaction. The data are the means ± SD from four determinations. $^{*}P < 0.05$, $^{**}P < 0.1$ (compared to A).

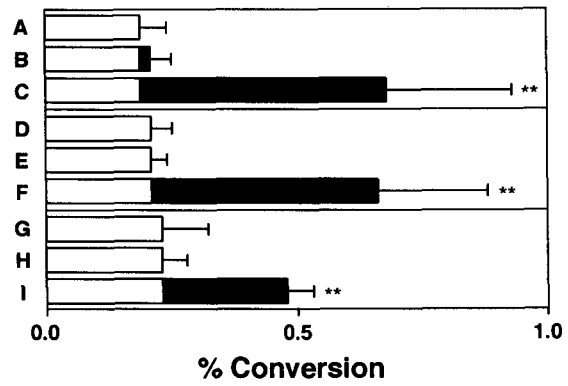

Fig. 5. [^{14}C]18:3(n-6) formed through CoA-dependent metabolic conversion of [^{14}C]18:2(n-6) in phospholipid and triacylglycerol fractions. The microsomal fraction was obtained from rat hepatocytes prelabeled with [^{14}C]18:2(n-6). The microsomal fraction was incubated with or without CoA (100 μM) in 50 mM Tris-HCl buffer (pH 7.3) containing 0.25% BSA and other materials as described in Materials and Methods. Total lipids were fractionated first by TLC (petroleum ether-diethyl ether-acetic acid 70:30:1 (v/v). The phospholipid fraction (the origin) was extracted from the silica gel and further fractionated by two-dimensional TLC as described in Materials and Methods. Percentages of the radioactivity located in phospholipid (the origin) and triacylglycerol fractions were 62% and 22%, respectively. (A)-(C), total phospholipids; (D)-(F), phosphatidylcholine; (G)-(I), triacylglycerols. (A), (D) and (G), before incubation; (B), (E) and (H), without CoA; (C), (F) and (I), with CoA. The data are the mean ± SD from four determinations. $^{**}P < 0.01$ (compared to that before incubation).

CoA-dependent modifications of the fatty acyl chains of membrane lipids. As demonstrated in **Figure 6,** free 18:2(n-6) did not undergo desaturation even in the presence of CoA when ATP and Mg^{2+} were omitted from the incubation mixture. The presence of CoA, ATP, and Mg^{2+} was absolutely required for the desaturation of free 18:2(n-6) either in the presence or absence (BSA alone) of gel-filtered cytosol. It should be noted, however, that the presence of gel-filtered cytosol was greatly preferable to that of BSA alone in the desaturation reaction when free 18:2(n-6) was used as the substrate. This is in contrast to the case of CoA-dependent/ATP-independent modification. We also confirmed that the presence of apyrase did not lower the overall enzyme reaction (**Fig. 7**). These observations indicate that free fatty acids are not involved in CoA-dependent/ATP-independent modification of fatty acyl chains of membrane lipids.

The effects of ATP and Mg^{2+} on CoA-dependent/ATP-independent modification of the fatty acyl moiety of membrane lipids were next studied. This system simulates the generally accepted machinery for the modification of fatty acyl moieties of membrane (phospho)lipids, i.e., the liberation of fatty acids by membranous and/or cytosolic lipolytic enzymes, the subsequent conversion of free fatty acids to acyl-CoA by acyl-CoA synthetase, and then enzymatic modification, followed by the reincorporation of the fatty acyl moiety into the parent molecules by acyltransferase(s). As illustrated in **Figure 8,** the addition of ATP and Mg^{2+} did not accelerate the desaturation reaction induced in the presence of CoA alone either in the presence or absence of gel-filtered cytosol. We also confirmed that the addition of Ca^{2+} (1 mM), which is known to stimulate phospholipase A_2 activity (25-29), did not dramatically accelerate the desaturation reaction (data not shown).

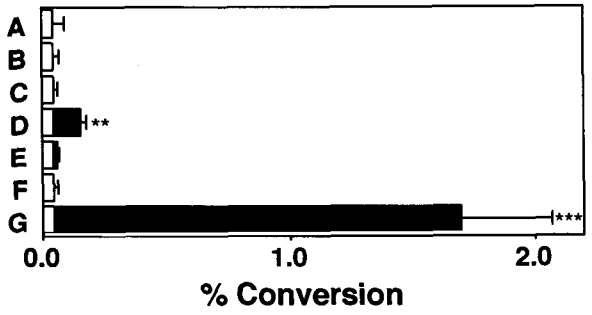

Fig. 6. Metabolic conversion of free [^{14}C]18:2(n-6) by liver microsomal fraction. The washed microsomal fraction (0.5 mg protein) obtained from the liver was incubated with [^{14}C]18:2(n-6) (100 μM) in 50 mM Tris-HCl buffer (pH 7.3) containing 0.25% BSA and other materials in the presence or absence of CoA (100 μM), ATP (5 mM) plus Mg^{2+} (5 mM) and gel-filtered cytosol (0.5 mg protein). The incubation was carried out for 30 min and then terminated by adding 10% KOH-methanol. Fatty acids were converted to fatty acid methyl esters and analyzed by HPLC. The rates of conversion of [^{14}C]18:2(n-6) to 18:3(n-6) were determined. (A), before incubation; (B), without CoA; (C), with CoA; (D), with CoA, ATP and Mg^{2+}; (E), with gel-filtered cytosol; (F), with CoA and gel-filtered cytosol; (G), with CoA, ATP, Mg^{2+}, and gel-filtered cytosol. The solid bars represent the rates of conversion during the enzyme reaction. The data are the mean ± SD from four determinations. $^{**}P < 0.01$, $^{***}P < 0.001$ (compared to A).

DISCUSSION

Isolated rat hepatocytes grown in a serum-free medium gradually metabolized various types of fatty acids after

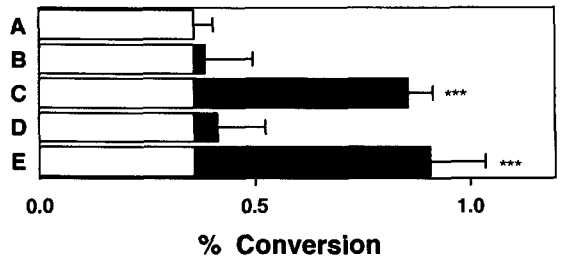

Fig. 7. Effects of apyrase on CoA-dependent metabolic conversion of [^{14}C]18:2(n-6) incorporated into microsomal lipids. The washed microsomal fraction was obtained from rat hepatocytes prelabeled with [^{14}C]18:2(n-6). The washed microsomal fraction was incubated with or without CoA (100 μM) in 50 mM Tris-HCl buffer (pH 7.3) at 37°C for 30 min. Fatty acyl chains were converted to fatty acid methyl esters and analyzed by HPLC as described in Materials and Methods. (A), before incubation; (B) without CoA; (C), with CoA; (D), without CoA and with apyrase (0.1 U/ml) plus Mg^{2+} (1 mM); (E) with CoA and apyrase plus Mg^{2+}. The data are the means ± SD from five determinations. $^{***}P < 0.001$ (compared to A).

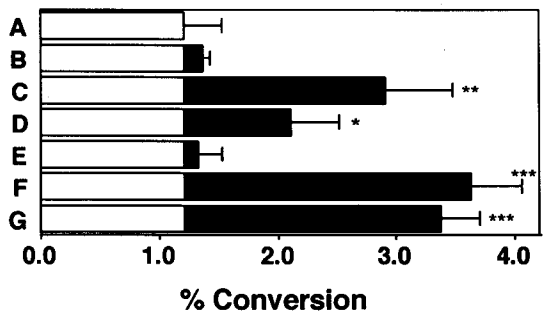

Fig. 8. Effects of ATP and Mg^{2+} on CoA-dependent metabolic conversion of [^{14}C]18:2(n-6) incorporated into microsomal lipids. The washed microsomal fraction was obtained from hepatocytes prelabeled with [^{14}C]18:2 for 60 min. The microsomal fraction (0.5 mg protein) was incubated in 50 mM Tris-HCl buffer containing BSA (0.25%) and other materials in the presence or absence of CoA (100 μM), ATP (5 mM), Mg^{2+} (5 mM) and gel-filtered cytosol (0.5 mg protein) for 30 min. The incubation was stopped by adding 10% KOH-methanol. Fatty acids were converted to fatty acid methyl esters and analyzed by HPLC. The rates of conversion of [^{14}C]18:2(n-6) to 18:3(n-6) were determined. (A) before incubation; (B) without CoA; (C) with CoA; (D) with CoA, ATP and Mg^{2+}; (E), with gel-filtered cytosol; (F), with CoA and gel-filtered cytosol; (G), with CoA, ATP, Mg^{2+}, and gel-filtered cytosol. The solid bars represent the rates of conversion during the enzyme reaction. The data are the mean ± SD from four determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (compared to A).

taking up these fatty acids within the cells (Fig. 1). Several lines of observation suggested that the modifications of fatty acids other than 18:0 predominantly take place after their incorporation into membrane lipids. It is well known that desaturation and chain elongation of fatty acids proceed in the form of acyl-CoA in mammalian tissues; fatty acyl moieties present in membrane lipids also require conversion to the corresponding acyl-CoA prior to undergoing enzymatic modifications. The following sequential reactions have been assumed to occur: the hydrolysis of lipid molecules, the subsequent conversion of free fatty acids to acyl-CoA by acyl-CoA synthetase, enzymatic modifications, and the reincorporation into lipid molecules by acyltransferase(s). The question arises as to whether liver cells really consume large amounts of ATP for such a deacylation-reacylation cycle even under conditions where the enzyme activities of desaturation and chain elongation are not high.

On the basis of the present results, we propose a simpler mechanism for the deacylation-reacylation cycle to modify membranous fatty acyl moieties. This system requires the presence of CoA but does not require ATP. It is apparent that ATP-independent transient acyl-CoA synthesis is involved in such CoA-dependent/ATP-independent modifications of fatty acyl residues of membrane lipids, as well as the CoA-dependent transacylation reaction (16). In fact, free fatty acids do not undergo any modifications in the absence of ATP and Mg^{2+} (Fig. 6). This is in good agreement with the previous observation that free fatty acids are not converted to acyl-CoA even in the presence of CoA when ATP and Mg^{2+} are omitted from the incubation mixture (Fig. 2). It is noticeable that the predominant fatty acyl moieties of acyl-CoA newly formed through ATP-independent acyl-CoA synthesis are 18:0, 18:2, and C20 polyunsaturated fatty acids (Table 1), which are good substrates for desaturation and/or chain elongation reactions, as shown in Fig. 1. Selective formation of acyl-CoA from membrane lipids for subsequent modifications would be rational compared with the classical system. Here we obtained clear evidence showing that the modifications of 18:0, 18:2(n-6), and C20 polyunsaturated fatty acids incorporated into membrane lipids take place when CoA is included in the reaction mixture even in the absence of ATP and Mg^{2+} (Fig. 3).

Some years ago, Pugh and Kates (30, 31) reported that [^{14}C]20:3(n-6) esterified in exogenously added phosphatidylcholine underwent direct desaturation without hydrolysis of the phosphatidylcholine molecule using rat liver microsomes. Microsomes from starved-refed rats exhibited higher enzyme activity and the presence of Triton X-100 was required for maximal enzyme activity. They also detected some activity for the desaturation of 1,2-di[^{14}C]oleoyl phosphatidylcholine but failed to detect activity for the desaturation of 1-acyl-2-[^{14}C]linoleoyl or 1-[^{14}C]stearoyl-2-acyl phosphatidylcholine. Consequently, this enzyme system may operate rather specifically for the desaturation of eicosatrienoyl residues. In the present study, we could not detect strong activity for the desaturation of 20:3(n-6) incorporated into membrane lipids when CoA was omitted from the reaction mixture (Fig. 3). The difference may be due to different experimental conditions. In any event, the direct desaturation system described above cannot be involved in the chain elongation reaction. On the other hand, the enzyme activities of CoA-dependent/ATP-independent modification of fatty acyl chains of membrane lipid demonstrated in the present study have more diverse fatty acid specificities (Fig. 3) and seem to have the capacity to account for various metabolic conversions, desaturation and chain elongation, observed in intact cells. Thus, it appears that CoA-dependent/ATP-independent modification of fatty acyl chains would be an advantageous mechanism for modifying membrane fatty acids (e.g., the conversion of 18:2(n-6) to 20:4(n-6)) in living cells than the direct desaturation system.

The presence of cytosol (untreated, gel-filtered, and dialyzed) or BSA in the reaction mixture potentiates CoA-dependent modification of 18:2(n-6) to 18:3(n-6) (Fig. 4). There seem to be two possible mechanisms. One is the direct stimulating effect of some cytosolic proteins on microsomal desaturase(s). Several investigators have already demonstrated that the addition of certain cytosolic proteins causes the acceleration of microsomal desaturase activities using several fatty acyl-CoA (or fatty acids plus CoA, ATP, and Mg^{2+}) as substrates (32-36). Considering the fact that acyl-CoA is transiently formed during CoA-

dependent modification of fatty acyl chains, it is possible that such cytosol proteins exert a stimulating effect directly on the enzymes, thereby accelerating the desaturation of 18:2 once esterified in membrane lipids. However, this is not the case, at least when BSA is used, because it has been shown that BSA does not stimulate the activity of Δ6 desaturase (33–36). Another possible mechanism relates to the increased availability of acyl-CoA for enzymes. We found that the presence of BSA resulted in the accumulation of acyl-CoA in the reaction mixture when microsomes were incubated with CoA, although the transient formation of acyl-CoA per se does not require the presence of BSA (16). BSA may interfere with the reincorporation of acyl moieties of acyl-CoA into acceptor lysophospholipids through its ability to bind acyl-CoA and lysophospholipids. In any event, the accumulation of acyl-CoA by BSA and possibly by cytosolic proteins also seems to be responsible, at least in part, for the enhanced enzymatic modification.

A striking observation is that the addition of ATP and Mg^{2+} did not dramatically enhance the desaturation reaction induced in the presence of CoA alone, even in the reconstituted system where gel-filtered cytosol was added (Fig. 8). It has already been demonstrated that either the microsomes or cytosol of the liver contain phospholipase activity (25–29). We also confirmed that both Ca^{2+}-independent and -dependent phospholipase A_2 activities are actually present in both the microsomal and cytosolic fractions used here (N. Kudo, unpublished data). It was assumed, therefore, that the following reactions take place: portions of membrane lipids are hydrolyzed to release free fatty acids, which, in turn, are converted to acyl-CoA by acyl-CoA synthetase in the presence of CoA, ATP, and Mg^{2+}. The resultant acyl-CoA could undergo subsequent enzymatic modifications before returning to the parent or other lipid molecules. However, the presence of ATP and Mg^{2+} failed to augment markedly the overall desaturation reaction either in the presence or absence of Ca^{2+}, suggesting that the CoA-dependent/ATP-independent modification pathway is sufficiently active to play a predominant role in the modification of fatty acyl chains of membrane lipids, especially phospholipids. In this regard, it is interesting to note that Mead and coworkers (37, 38) have already suggested that modifications of membranous fatty acids may occur within the membrane without facile equilibration with the external free fatty acid pool, although they did not examine the possible involvement of a CoA-dependent/ATP-independent reaction.

Because the conditions in the reconstituted system used here are not the same as those in living cells, it is not easy to assess the relative importance of individual pathways implicated in the modifications of fatty acyl chains of membrane lipids in vivo. Living cells are highly organized dynamic systems and it is important to take into account possible involvement or contribution of other organelles and possible compartmentalization of various metabolic processes. In addition, it remains unclear whether the modification of fatty acyl chains of triacylglycerol (Fig. 5) proceeds in a way similar to that for phospholipids. Recently, we confirmed that endogenous phosphatidylcholine and phosphatidylinositol are the predominant acyl donors in the ATP-independent acyl-CoA synthesis from membrane lipids (T. Sugiura, unpublished data). There is a possibility, therefore, that the acyl-CoA formed from these phospholipids is modified and then incorporated into diacylglycerol to yield triacylglycerol. However, the possibility that acyl-CoA is formed directly from triacylglycerol cannot be ruled out. Thus, further studies are required to understand the details of the mechanism underlying the modification of fatty acyl moieties of membrane lipids in living cells.

In summary, we have proposed here a novel mechanism for the modification of fatty acyl moieties of membrane lipids, especially membrane phospholipids (**Fig. 9**). Certain types of fatty acyl chains of membrane phospholipids are continuously being transiently converted to acyl-CoA without consumption of ATP. The resultant acyl-CoA undergoes enzymatic modifications when enzyme activities for desaturation and/or chain elongation are increased, to modify the fatty acid composition of mem-

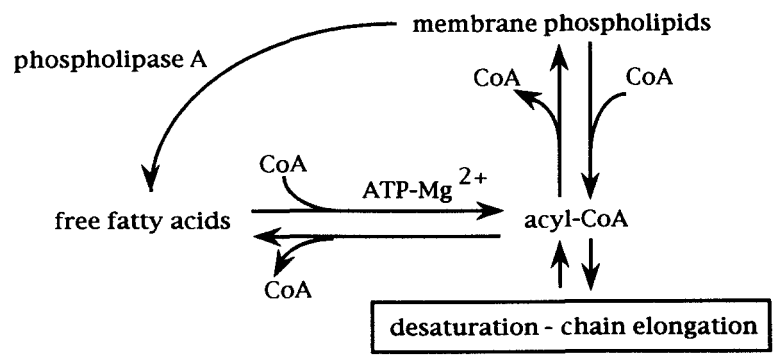

Fig. 9. Proposed mechanism for the metabolic conversion of fatty acyl moieties of membrane phospholipids in mammalian tissues.

brane phospholipids. This system is effective in modifying fatty acids esterified in membrane phospholipids. On the other hand, free fatty acids derived from extracellular pools or those liberated from cellular lipids are converted to the corresponding acyl-CoA with the consumption of ATP and then undergo enzymatic modification prior to incorporation into acceptor lipid molecules. These two pathways thus appear to act in concert in the modification of esterified and nonesterified long chain fatty acids in living cells.

We are grateful to Miho Tanaka and Yukiko Kimura for their technical assistance. This study was supported in part by a Grant-in-Aid (No. 0580859) from the Ministry of Education, Culture and Science of Japan.

Manuscript received 8 January 1994 and in revised form 17 October 1994.

REFERENCES

1. White, D. A. 1973. The phospholipid composition of mammalian tissues. *In* Form and Function of Phospholipids. G. B. Ansell, J. N. Hawthorne, and R. M. C. Dawson, editors. Elsevier, Amsterdam. 441–482.
2. Pace-Asciak, C., and E. Gränstrom, editors. 1983. Prostaglandins and Related Substances. Elsevier, Amsterdam.
3. Tinoco, J. 1982. Dietary requirements and functions of α-linolenic acid in animals. *Prog. Lipid Res.* **21:** 1–45.
4. Brenner, R. R. 1971. The desaturation step in the animal biosynthesis of polyunsaturated fatty acids. *Lipids.* **6:** 567–575.
5. Bernert, J. T., Jr., and H. Sprecher. 1975. Studies to determine the role rates of chain elongation and desaturation play in regulating the unsaturated fatty acid composition of rat liver lipids. *Biochim. Biophys. Acta.* **398:** 354–363.
6. Sprecher, H. 1981. Biochemistry of essential fatty acids. *Prog. Lipid Res.* **20:** 13–22.
7. Brenner, R. R. 1981. Nutritional and hormonal factors influencing desaturation of essential fatty acids. *Prog. Lipid Res.* **20:** 41–47.
8. Spector, A. A., S. N. Mathur, T. L. Kaduce, and B. T. Hyman. 1981. Lipid nutrition and metabolism of cultured mammalian cells. *Prog. Lipid Res.* **19:** 155–186.
9. Hagve, T. A., and B. O. Christophersen. 1983. Linolenic acid desaturation and chain elongation and rapid turnover of phospholipid n-3 fatty acids in isolated rat liver cells. *Biochim. Biophys. Acta.* **753:** 339–349.
10. Jeffcoat, R., and A. T. James. 1984. The regulation of desaturation and elongation of fatty acids in mammals. *In* Fatty Acid Metabolism and Its Regulation. S. Numa, editor. Elsevier, Amsterdam. 85–112.
11. Holman, R. T. 1986. Control of polyunsaturated acids in tissue lipids. *J. Am. Coll. Nutr.* **5:** 183–211.
12. Rosenthal, M. D. 1987. Fatty acid metabolism of isolated mammalian cells. *Prog. Lipid Res.* **26:** 87–124.
13. Brenner, R. R. 1989. Factors influencing fatty acid chain elongation and desaturation. *In* The Role of Fats in Human Nutrition. 2nd ed. A. J. Vergroesen, and M. Crawford, editors. Academic Press, New York. 45–79.
14. Norum, K. R., E. N. Christiansen, B. O. Christophersen, and J. Bremer. 1989. Metabolic and nutritional aspects of long-chain fatty acids of marine origin. *In* The Role of Fats in Human Nutrition. 2nd ed. A. J. Vergroesen, and M. Crawford, editors. Academic Press, New York. 117–149.
15. Cook, H. W. 1991. Fatty acid desaturation and chain elongation in eucaryotes. *In* Biochemistry of Lipids, Lipoproteins and Membranes. D. E. Vance, and J. E. Vance, editors. Elsevier, Amsterdam. 141–169.
16. Sugiura, T., Y. Masuzawa, and K. Waku. 1988. Coenzyme A-dependent transacylation system in rabbit liver microsomes. *J. Biol. Chem.* **263:** 17490–17498.
17. Stymne, S., and A. K. Stobart. 1985. Involvement of acyl exchange between acyl-CoA and phosphatidylcholine in the remodeling of phosphatidylcholine in microsomal preparation of rat lung. *Biochim. Biophys. Acta.* **837:** 239–250.
18. Seglen, P. O. 1976. Preparation of isolated rat liver cells. *Methods Cell Biol.* **13:** 29–83.
19. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
20. Kates, M. 1986. Techniques of Lipidology. Isolation, Analysis and Identification of Lipids, 2nd ed. Elsevier, Amsterdam.
21. Kudo, N., Y. Nakagawa, and K. Waku. 1990. Effects of zinc deficiency on the fatty acid composition and metabolism in rats fed a fat-free diet. *Biol. Trace Element Res.* **24:** 49–60.
22. Aveldano, M. I., M. VanRollins, and L. A. Horrocks. 1983. Separation and quantitation of free fatty acids and fatty acid methyl esters by reverse phase high pressure liquid chromatography. *J. Lipid Res.* **24:** 83–93.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
24. Mahfouz, M. M., A. J. Valicenti, and R. T. Holman. 1980. Desaturation of isomeric *trans*-octadecenoic acids by rat liver microsomes. *Biochim. Biophys. Acta.* **618:** 1–12.
25. Nachbaur, J., A. Colbeau, and P. M. Vignais. 1972. Distribution of membrane-confined phospholipases A in the rat hepatocyte. *Biochim. Biophys. Acta.* **274:** 426–446.
26. Newkirk, J. D., and M. Waite. 1973. Phospholipid hydrolysis by phospholipases A_1 and A_2 in plasma membranes and microsomes of rat liver. *Biochim. Biophys. Acta.* **298:** 562–576.
27. Lumb, R. H., and K. F. Allen. 1976. Properties of microsomal phospholipases in rat liver and hepatoma. *Biochim. Biophys. Acta.* **450:** 175–184.
28. Van den Bosch, H. 1980. Intracellular phospholipases A. *Biochim. Biophys. Acta.* **604:** 191–246.
29. Waite, M. 1987. The Phospholipases. Plenum Press, New York.
30. Pugh, E. L., and M. Kates. 1977. Direct desaturation of eicosatrienoyl lecithin to arachidonoyl lecithin by rat liver microsomes. *J. Biol. Chem.* **252:** 68–73.
31. Pugh, E. L., and M. Kates. 1979. Membrane-bound phospholipid desaturases. *Lipids.* **14:** 159–165.
32. Catala, A., A. M. Nervi, and R. R. Brenner. 1975. Separation of a protein factor necessary for the oxidative desaturation of fatty acids in the rat. *J. Biol. Chem.* **250:** 7481–7484.
33. Jeffcoat, R., A. P. Dunton, and A. T. James. 1978. Evidence for the different responses of Δ9 and Δ6 and Δ5-fatty acyl-CoA desaturase to cytoplasmic proteins. *Biochim. Biophys. Acta.* **528:** 28–35.
34. Leikin, A. I., A. M. Nervi, and R. R. Brenner. 1979. Lipid binding properties of a factor necessary for linoleic acid desaturation. *Lipids.* **14:** 1021–1026.
35. Leikin, A. I., and R. R. Brenner. 1986. Regulation of linoleic acid Δ6-desaturation by a cytosolic lipoprotein-like fraction in isolated rat liver microsomes. *Biochim. Biophys.*

Acta. **876:** 300–308.
36. Leikin, A. I., and R. R. Brenner. 1989. Microsomal Δ5 desaturation of eicosa-8,11,14-trienoic acid is activated by a cytosolic fraction. *Lipids.* **24:** 101–104.
37. Aeberhard, E. E., M. Gan-Elepano, and J. F. Mead. 1981. Metabolism of fatty acids in rat brain microsomal membranes. *Lipids.* **16:** 705–713.
38. Gan-Elepano, M., E. Aeberhard, and J. F. Mead. 1981. On the mechanism of fatty acid transformations in membranes. *Lipids.* **16:** 790–795.