[Chem. Pharm. Bull. 24(1) 46 — 51 (1976)]

UDC 577.158.02.03 : 543.544.25.062

Some Factors affecting Chain Elongation of Palmityl-CoA in Rat Liver Microsomes[1]

Mitsuo Nakagawa,[2a] Yoichi Kawashima, and Mitsuru Uchiyama[2b]

Pharmaceutical Institute, Tohoku University[2]

(Received May 8, 1975)

The chain elongation of labeled palmityl-CoA by rat liver microsomes was studied by radio-gas liquid chromatography after the incubation of the acid in a medium containing malonyl-CoA, β-mercaptoethanol, reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), KCN and sucrose under anaerobic conditions.

The activity of chain elongation of palmityl-CoA to stearic acid was increased by refeeding starved rats. The activity in liver microsomes of refed rats was approximately twice greater than that of normal rats. Lipid phosphorus contents in liver microsomes of normal (15.4±0.8 μg P/mg protein) and refed rats (15.3±1.6 μg P/mg protein) were unchanged. However, the reaction was stimulated by the addition of sonicated dispersion of phosphatidylcholine. The stimulatory effect of the dispersion was more remarkable for refed rats than for normal ones.

On the other hand, the chain elongation activity of palmityl-CoA was decreased by acetone extraction or phospholipase C treatment of liver microsomes of refed rats. The decreased activity was partially or completely restored by the addition of sonicated dispersion of phosphatidylcholine.

These results suggest that phospholipids may play an important role for chain elongation reaction of fatty acids in liver microsomes of rats.

Introduction

It is now recognized that two different enzyme systems play a role during the synthesis of fatty acids in rat liver microsomes.[3] A distinction can be made between the synthesis *de novo* and the chain elongation of fatty acids. The microsomal chain elongation requires malonyl-CoA rather than acetyl-CoA as active donor for the 2-fragments.[4] The rate of the reaction is markedly affected by the chain length and degree of unsaturation of fatty acyl-CoA esters used as substrate,[5] the amounts of reduced pyridine nucleotides and adenosine 5′-triphosphate (ATP) as cofactor[3] and the coexisting saturated and unsaturated fatty acids.[6]

Some microsomal enzymes, acyl–CoA–glycerol-3-phosphate acyltransferase, palmityl–CoA synthetase[7] and uridine 5′-diphosphate (UDP)-glucuronyl transferase[8] require phospholipids for normal catalytic activity.

In the present paper, evidence is presented for the effect of sonicated dispersion of phosphatidylcholine on the chain elongation of palmityl–CoA in native– and acetone extracted–liver microsomes of normal and refed rats under anaerobic condition.

1) This work was presented at the 95 th Annual Meeting of Pharmaceutical Society of Japan, Nishinomiya, April 1975.
2) Location: *Aobayama, Sendai*; *a*) Present address: *Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto*; *b*) *National Institute of Hygienic Sciences, Kamiyoga-1-chome, Setagaya, Tokyo.*
3) C. Landriscina, G.V. Gnoni, and E. Quagliariello, *Biochim. Biophys. Acta*, **202**, 405 (1970).
4) W. Seubert and E.R. Podack, *Mol. Cell. Biochem.*, **1**, 29 (1973).
5) D.H. Nugteren, *Biochim. Biophys. Acta*, **106**, 280 (1965).
6) H. Mohrhauer, K. Christiansen, M.V. Gan, M. Deubig, and R.T. Holman, *J. Biol. Chem.*, **242**, 4507 (1967).
7) R. Tzur and B. Shapiro, *Israel J. Med. Sci.*, **5**, 971 (1969).
8) A.B. Graham and G.C. Wood, *Biochem. Biophys. Res. Commun.*, **37**, 567 (1969).

Materials and Methods

Chemicals——Palmityl-1-^{14}C-CoA (58.2 mCi/mM) was obtained from New England Nuclear Corp (Boston, Mass., U.S.A.). Malonyl-CoA and palmityl-CoA were purchased from Nutritional Biochemicals Co. (Cleveland, Ohio, U.S.A.); NADH, NADPH and bovine serum albumin from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Phosphatidylcholine was prepared from egg yolk by the method of Faure[9] and purified by the same procedure as described previously.[10]

Animals——Female albino rats of the Wistar strain, weighing 80—120 g, were used. The animals were fed *ad libitum* on a commercial diet obtained from Oriental Yeast Company (Tokyo, Japan). Some animals were starved for 48 hr and then fed the commercial diet *ad libitum* for 24 hr. These rats are referred to as "refed rats."

Preparation of Enzyme——The rats were killed by cervical fracture and livers were excised and homogenized with 3 volumes of cold 0.25 M sucrose solution. Cellular debris, nuclei and mitochondrial fractions were removed from the whole homogenate by centrifugation at 12000 *g* for 15 min. From the supernatant fluid, a microsomal fraction was obtained by centrifugation at 100000 *g* for 60 min. The microsomal pellet was suspended in an aliquot volume of 0.25 M sucrose and then sedimented at 100000 *g* for 60 min. The pellet was resuspended in an aliquot of 0.1 M phosphate buffer (pH 7.4) containing 0.25 M sucrose. The suspension was used as enzyme source.

Preparation of Acetone Extracted-liver Microsomes——Liver microsomes were mixed with 20 volumes of cold acetone which had been cooled to $-15°$.[11] The mixture was allowed to stand for 10 min at $-15°$ and was then centrifuged at 9000 *g* for 10 min at 4°. The precipitate was washed with cold acetone and recentrifuged. After the supernatant was discarded, and the precipitate was resuspended in cold ether, collected by centrifugation and then dried overnight at 4° in a vacuum desicator containing $CaCl_2$. Prior to use acetone powder of liver microsomes, the powder was dissolved in 0.1 M phosphate buffer (pH 7.4) which had been cooled to 4°. The clear supernatant fraction was used as enzyme source. The protein contents in the enzyme solution were determined by the procedure described by Lowry, *et al.*[12] with crystalline bovine serum albumin as standard.

Assay for Chain Elongation of Fatty Acids——The standard incubation mixture contained 45 μM palmityl-CoA, 3.4 μM palmityl-1-^{14}C-CoA, 100 μM malonyl-CoA, 6 mM β-mercaptoethanol, 3 mM NADH, 3 mM NADPH, 25 mM sucrose, 1 mM KCN and microsomes (protein, 0.8—1.1 mg). The final volume was adjusted to 1 ml with 0.1 M phosphate buffer (pH 7.4). Incubation was carried out at 37° for 8 min under N_2 with constant shaking. At the end of the incubation time, the reaction was stopped by the addition of 1 ml of 10% KOH-methanolic solution and then heated at 70° for 30 min. After acidified with 6N–HCl, extraction of lipids and isolation of fatty acids were made by the same procedure as described previously.[13]

Analysis of Fatty Acid Mixtures by Radio-gas Liquid Chromatography——Total fatty acids extracted from the incubation medium were methylated and then analyzed by means of radio-gas liquid chromatography as described previously.[14] Gas-liquid chromatographical analysis was performed by using a Shimazu GC-3AH gas-liquid chromatographical instrument. The column (column length; 2.25 m) coated 80—100 mesh celite 545 which was coated with 20% (w/w) diethylene glycol succinate polyester. The operation temperature was 186—190°. The radioactivity in each fatty acid was determined by Shimazu RID-2A radioisotope detector connected with gas-liquid chromatography mentioned above. The rate of chain elongation of fatty acid was mainly calculated from the peak area of radioactivity of each fatty acid on radiochromatogram. In some experiments, the desired methyl esters were directly collected from gas-liquid chromatography according to the procedure of Aeberhard and Menkes,[15] and measured the radioactivity in a toluene-2,5-diphenyloxazole (PPO)-1,4-bis [2-(5-phenyloxazolyl)]-benzene (POPOP) scintillation mixture using a Tricarb liquid scintillation spectrometer.[16]

Preparation of Sonicated Dispersion of Phosphatidylcholine——The sonicated dispersion of phosphatidylcholine was prepared in the same manner as described previously.[16] It was confirmed by thin layer chromatography that no degradation of phosphatidylcholine to lysophosphatidylcholine occurred during the sonication. The method used for the determination of lipid phosphorus in the dispersion and in the total lipids extracted from liver microsomes was previously described.[16]

Results and Discussion

It has been reported that the most effective system for the chain elongation of fatty acids is liver microsomes plus malonyl-CoA with cofactors, NADH and NADPH under aerobic

9) M. Faure, *Bull. Soc. Chim. Biol.*, 32, 503 (1950).
10) M. Nakagawa and M. Uchiyama, *Biochem. Pharmacol.*, 23, 1641 (1974).
11) R.K. Morton, "Method in Enzymology," Vol. 1, Academic Press, New York, 1955, p. 34.
12) O.H. Lowry, H.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, 193, 265 (1951).
13) M. Uchiyama, M. Nakagawa, and S. Okui, *J. Biochem.*, 62, 1 (1967).
14) M. Nakagawa and M. Uchiyama, *J. Biochem.*, 63, 684 (1968).
15) E. Aeberhard and J.H. Menkes, *J. Biol. Chem.*, 243, 3834 (1968).
16) M. Nakagawa and T. Nishida, *Biochim. Biophys. Acta*, 296, 577 (1973).

condition[3] and that under anaerobic condition, the oxidative desaturation reaction can be eliminated from the chain elongation reaction.[6]

In the present paper, the effect of some factors on the chain elongation of palmityl-CoA by rat liver microsomes under anaerobic condition was studied by the use of partially modified procedure of Landriscina, *et al.*[3] as indicated in the text.

We have confirmed that under the present experimental conditions, the labeled fatty acid derived from palmityl-1-^{14}C-CoA by normal and refed rats was only stearic acid and also that the recovery of ^{14}C in palmityl-1-^{14}C-CoA was over 95% of the initial radioactivity. Thus, the occurrence of oxidative degradation of substrate and product was excluded.

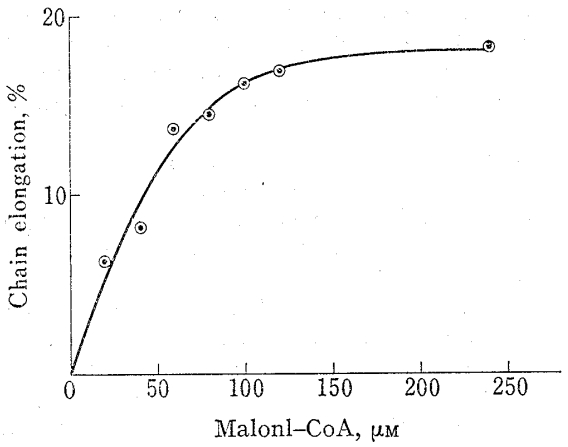

Fig. 1. Effect of Malonyl-CoA Concentration on Chain Elongation by Rat Liver Microsomes

The assay system contained in a final volume of 1 ml; 45 μM palmityl-CoA, 3.4 μM palmityl-1-^{14}C-CoA (0.2 μci), 100 μM malonyl-CoA, 6 mM β-mercaptoethanol, 3 mM NADH, 3 mM NADPH, 25 mM sucrose, 1 mM KCN and 1.1 mg liver microsomal protein of normal rats. The incubation was carried out at 37° under N_2 with constant shaking. The percentage of chain elongation of fatty acid was calculated from the peak area of radioactivity of each fatty acid on radiochromatogram.

Furthermore, under the present experimental conditions, the chain elongation of labeled palmityl-CoA to stearic acid by liver microsomes was linear during the first 10 min and also linearly increased up to 1.5 mg of microsomal protein. As shown in Figs. 1 and 2, the chain elongation of labeled palmityl-CoA was dependent on the concentrations of malonyl-CoA and palmityl-CoA in the incubation medium. The reaction reached to a plateau at the concentration of approximately 100 μM of malonyl-CoA and also at the concentration of approximately 30 μM of palmityl-CoA, but not significantly affected up to about 50 μM of palmityl-CoA. From the results, we used 45 μM of malonyl-CoA and 100 μM of palmityl-CoA as described in "Materials and Methods."

In general, fatty acid metabolism in rat liver is significantly affected by physiological conditions. In the course of this investigation, Sprecher has reported that when the rats fasted for 72 hr were fed the chow diet for 24 hr, the rate of chain elongation of palmitic acid was about 4 times greater than that for the nonfasted controls.[17] We also independently observed in Table I that the activity (27.0±1.3%) of chain elongation of palmityl-CoA to stearic acid in liver microsomes of refed rats was approximately twice greater than for that (14.2±3.8%) of normal rats. The difference in responce to refeeding of the chain elongation activity between the results obtained by Sprecher and our laboratory may be mainly due to the starvation period.

Table I. The Activity of Chain Elongation of Palmityl-CoA in Liver Microsomes of Normal and Refed Rats

Microsomes	Chain elongation, % average ±SD
Normal rats	14.2±3.8(7)
Refed rats	27.0±1.3(4)

The incubation conditions were the same as in Fig. 1 except for the use of 1 mg of microsomal protein of normal and refed rats. The values in parentheses indicate a number of experiments.

17) H. Sprecher, *Biochim. Biophys, Acta*, **360**, 113 (1974).

Table II. Effect of Sonicated Dispersion of Phosphatidylcholine on Chain Elongation of Palmityl-CoA in Liver Microsomes of Normal and Refed Rats

Condition of rats	Exptl. No.	Chain elongation, %	
		PC (−)	PC (+)
Normal	I	15.5	22.5(145)
Refed	I	25.4	39.8(157)
	II	26.7	42.7(160)
	III	29.0	48.0(166)

One mg of liver microsomes and various amounts of the dispersion were preincubated at 0° for 60 min and then incubated with cofactors and substrates at 37° for 8 min. The amounts of phosphatidylcholine (PC) used were 314 μg, 716 μg and 476 μg per incubation medium for experiments I, II and III, respectively. The other incubation conditions were the same as in Fig. 1. The Values in parentheses represent percent change from PC (−).

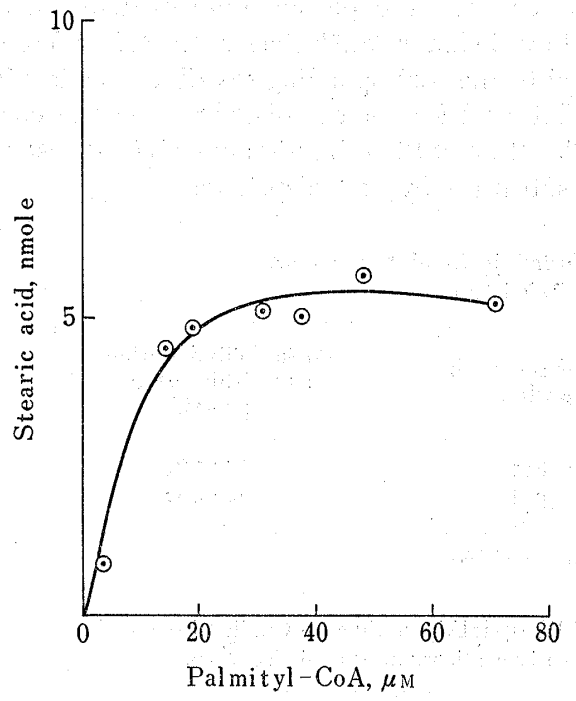

Fig. 2. Effect of Palmityl-CoA Concentration on Chain Elongation by Rat Liver Microsomes

The incubation conditions were the same as in Fig. 1 except for the addition of 1.0 mg of microsomal protein.

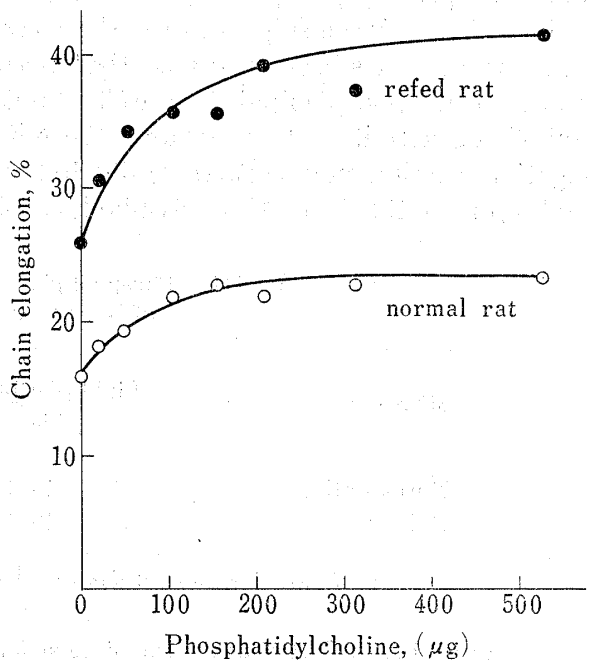

Fig. 3. Effect of Sonicated Dispersion of Phosphatidylcholine on Chain Elongation of Palmityl-CoA in Liver Microsomes of Normal and Refed Rats

The incubation conditions were the same as in Fig. 1 except for the addition of 1 mg of liver microsomal protein of normal and refed rats and various amounts of sonicated dispersion of phosphatidylcholine.

The enzyme catalyzing the chain elongation and desaturation are located in the same subcellular fraction in rat liver and show the same cofactor requirements.[4] In addition, the oxidation of NADPH coupled to the desaturation of stearyl-CoA in liver microsomes possesses a rather specific requirement of phospholipids.[18] Therefore, it is of interest to investigate the effect of phospholipid on the chain elongation reaction. Table II shows the effect of sonicated dispersion of phosphatidylcholine on the chain elongation in liver microsomes prepared from normal and refed rats. The extent of chain elongation was stimulated approximately 45% for normal rats and 70% for refed ones by the addition of the dispersion. To further study the effect of phosphatidylcholine on chain elongation, liver microsomes of normal and refed rats were incubated with various amounts of sonicated dispersion of phosphatidyl-

18) P.D. Jones and S.J. Wakil, *J. Biol. Chem.*, **242**, 5267 (1967).

choline. As shown in Fig. 3, the extent of chain elongation was gradually enhanced by the addition of increasing amounts of the dispersion and then reached to a plateau at the concentration of approximately 150 μg and 250 μg of phosphatidylcholine/incubation medium for normal and refed rats, respectively. The stimulatory effect of the dispersion was more remarkable for refed rats than for normal rats.

Allmann, *et al.*[19] have reported that liver phospholipid contents decrease to about two-third of its normal level when a balanced diet is fed to rats for 23 hr after starvation of 48 hr. However, as shown in Table III, total lipid phosphorus contents in liver microsomes of both normal and refed rats were unchanged. Although no conclusive information is now available on the difference of the extent of stimulation of the chain elongation activity in liver microsomes of normal and refed rats by the dispersion, we have observed that the chain elongation activity could not be remarkably stimulated by the addition of α-glycerophosphate and lysophosphatidylcholine instead of phosphatidylcholine. Accordingly, the amount of some specific phospholipids, especially phosphatidylcholine, to the enzyme may play an important role on this reaction. The relationship between the enzyme activity and phospholipid contents in rat liver microsomes has been most recently reported by Belina, *et al.*,[20] that is, the sex difference in some specific phospholipids may well be related to the corresponding sex difference in drug metabolizing activity. Furthermore, the stimulatory effect of the dispersion on the chain elongation reaction may be partially due to the elevation of the formation of enzyme-substrate complex or to the increase in availability of substrate by the dispersion.

Table III. Phospholipid Contents in Liver Microsomes of Normal and Refed Rats

Microsomes	Lipid phosphorus contents μg/mg protein	Phospholipid contents as lecithin, μg/mg protein
Normal rats	15.4±0.8(4)	392±21
Refed rats	15.3±1.6(4)	389±38

The values in parentheses indicate a number of experiments.

Table IV. Effect of Sonicated Dispersion of Phosphatidylcholine on Chain Elongation of Palmityl-CoA in Acetone Extracted Liver Microsomes of Refed Rats

Addition	Chain elongation, %	
	I	II
Acetone powder of liver microsomes	3.0(10.3)	2.6(8.9)
+phosphatidylcholine (82 μg)	5.4(18.6)	—
+phosphatidylcholine (420 μg)	7.1(24.4)	—
+phosphatidylcholine (794 μg)	—	14.4(49.6)
Native liver microsomes	29.0(100)	29.0(100)

One mg of acetone extracted-liver microsomes and the dispersion were preincubated at 0° for 60 min and then incubated with cofactors and substrates at 37° for 8 min. The other incubation conditions were the same as in Fig. 1. The phosphorus contents of native and acetone extracted liver microsomes were 14.9 μg and 7.2 μg per mg protein, respectively.

On the other hand, glycerides, fatty acids and a part of phospholipids in liver microsomes can be extracted by aqueous acetone. As shown in Table IV, the acetone extracted-liver microsomes were decreased their ability to elongate palmityl-CoA to stearate. The activity

19) D.W. Allmann, D.D. Hubbard, and D.M. Gibson, *J. Lipid Res.*, **6**, 63 (1965).
20) H. Belina, S.D. Cooper, R. Farkas, and G. Feuer, *Biochem. Pharmacol.*, **24**, 301 (1975).

was partially restored by the addition of sonicated dispersion of phosphatidylcholine as reported by the observations of Jones, *et al.*[21] in which liver microsomes extracted with aqueous acetone lose the ability to desaturate stearyl-CoA to oleate and in which the desaturase activity of these particles is partially restored by the addition of phosphatidylcholine. In addition, as shown in Table V, the chain elongation activity in liver microsomes was decreased by phospholipase C treatment. The decreased activity was accompanied by the decrease in microsomal phospholipid contents and recovered by the addition of the dispersion.

TABLE V. Effect of Phospholipase C Treatment on Chain Elongation of Palmityl-CoA in Liver Microsomes of Refed Rats

Enzyme preparation	Lipid phosphorus contents μg/mg protein	Chain elongation %
Nativemicrosomes	15.0	20.1
+phosphatidylcholine (554 μg)		29.0
Phospholipase C-treated microsomes	11.5	9.3
+Phosphatidylcholine (554 μg)		32.4

One mg of liver microsomal protein was incubated with 0.04 mg of phospholipase C (from Cl. Welchii, approx. 5 units per mg protein, Sigma Chemical Co.), 3 μmoles of β-mercaptoethanol, 0.7 μmole of $CaCl_2$ and 25 μmoles of sucrose in 0.39 ml of tris buffer (pH 7.4) at 23° for 5 min. The reaction was stopped by the addition of 10 μmoles of ethylenediamine tetraacetic acid. After phospholipase C treatment, the chain elongation activity was measured the same conditions in Fig. 1. The combination of the dispersion and phospholipase C treated microsomes was carried out by reincubation for 60 min at 0°.

These results suggest that the changes in chain elongation activity of palmityl-CoA in liver microsomes were accompanied by parallel alterations in microsomal phospholipid contents. Furthermore, experiments are now in progress to study the mechanism of the stimulatory effect on chain elongation reaction by the dispersion.

21) P.D. Jones, P.W. Holloway, R.O. Peluffo, and S.J. Wakil, *J. Biol. Chem.*, **244**, 744 (1969).