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In Vivo Studies on the de Novo Synthesis of Molecular Species of Rat Lung Lecithins

TAKAHARU MORIYA and HIDEO KANOH

The Third Department of Internal Medicine and The Fist Division,† Department of Biochemistry, Sapporo Medical College, Sapporo*

MORIYA, T. and KANOH, H. *In Vivo Studies on the de Novo Synthesis of Molecular Species of Rat Lung Lecithins.* Tohoku J. exp. Med., 1974, **112** (3), 241–256 —— In vivo incorporation of intrafemorally injected [9, 10-$^3H_2$]-palmitic acid and [2-^{3}H]-glycerol into molecular species of rat lung glycerolipids was studied in relatively early periods after injection (from 2 to 30 min). The pool size of 1,2-diacylglycerols was also determined in the tissue. When [9,10-$^3H_2$]-palmitic acid was injected, approximately 60% of the radioactivity was distributed in 1,2-disaturated species of phosphatidic acids and 1,2-diacylglycerols in 2 min, and this labeling pattern was reflected on the synthesis of lecithin species, although the calculated turnover of molecular species of lecithins and the results obtained with [2-^{3}H]-glycerol injection suggested that the pathway of synthesis de novo could not be regarded as being fully responsible for the formation of dipalmitoyl lecithin in rat lung. Analysis of the distribution of radioactivity in the 1- and 2-positions of the formed glycerolipids after administration of [9,10-$^3H_2$]-palmitic acid showed that approximately 75% of the incorporated radioactivity was located in the 2-position of dipalmitoyl lecithin during the present experimental periods, while the ratio of the distribution was almost 1:1 in disaturated species of 1,2-diacylglycerols. This phenomenon could reasonably explain the discrepancy observed between the experimental results with [9, 10-$^3H_2$]-plamitic acid and [2-^{3}H]-glycerol, suggesting that there exists other unknown mechanism than de novo synthesis to introduce palmitic acid of a higher specific radioactivity to the 2-position of dipalmitoyl lecithin. ———— 1,2-diacylglycerols; dipalmitoyl lecithin

Naturally occurring lecithins in most animal tissues have been found to be esterified principally with a saturated fatty acid in the 1-position and with an unsaturated in the 2-position, while the mammalian lung tissues have been confirmed to contain typically large amounts of dipalmitoyl lecithin (Kanoh 1967; Veerkamp et al. 1962; Montfoort et al. 1971). Various metabolic routes leading to the formation of this specific lecithin species have been suggested by many investigators in the lung tissues. Although the recent progress in the studies of the metabolism of molecular species of glycerophospholipids has revealed distinct characteristics of various biosynthetic pathways to form specific phospholipid

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species, especially in rat liver (Arvidson 1968; Kanoh 1969, 1971; Åkesson 1970; Åkesson et al. 1970 a, b), the metabolic origin of dipalmitoyl lecithin has not yet been completely understood. Frosolono et al. (1971) and Vereyken et al. (1972) suggested the possible participation of acyl-coenzyme A: lysolecithin acyltransferase (Lands 1960) in forming dipalmitoyl lecithin from the data obtained by comparative studies with the liver and lung microsomal fractions.

On the other hand, Akino et al. (1972), Abe et al. (1972, 1973) and Abe and Akino (1972) presented evidence to show that the transacylation mechanism between two molecules of 1-palmitoyllysolecithin, first reported by Erbland and Marinetti (1965), can contribute to the formation of this specific species of rat lung lecithins. The contribution of N-methylation of phosphatidylethanolamines (Bremer and Greenberg 1959) to the synthesis of this compound was also suggested by Morgan (1969) in dog lung.

Although some features of de novo synthesis of rat lung lecithins have already been briefly reported (Vereyken et al. 1971), detailed informations on this pathway are still lacking.

In the present investigation incorporation of labeled palmitic acid and glycerol into molecular species of rat lung lecithins and the relating precursors was studied in vivo in order to elucidate the characteristics of the de novo synthesis in forming molecular species of rat lung lecithins.

Materials and Methods

Materials

[9,10-$^3H_3$]-palmitic acid (specific radioactivity: 500 mCi/mM) and [2-^{3}H]-glycerol (380 mCi/mM) were purchased from The Radiochemical Centre, Amersham, England. Bovine serum albumin (Fraction V) purchased from Kishida Chem. Co. (Japan) was defatted by the method of Goodman (1957). [9,10-$^3H_2$]-palmitic acid was complexed with albumin according to Åkesson et al. (1970b). Snake venom (*Crotalus adamanteus*), phospholipase C (*Clostridium welchii*), cabbage phospholipase D and hog pancreatic lipase were the products of Sigma Chem. Co. (U.S.A.). Na-salts of phosphatidic acids were prepared by phospholipase D hydrolysis of purified rat lung lecithins by the method of Okuyama et al. (1971). 1,2-Diacylglycerols were prepared from lung or liver lecithins as previously described (Kanoh 1970; Kanoh and Ohno 1973b) and were used as a carrier. All solvents were glass-distilled and no antioxidants were used.

Treatment of animals, injection procedure and extraction of lung lipids

Male or female Wistar rats weighing 180–220 g were used after overnight fasting. The following procedure was essentially the same as reported by Åkesson et al. (1970b). 0.2 ml of 0.9% saline containing 100 μCi of [9,10-$^3H_2$]-palmitic acid and 12 mg of albumin or 0.25 ml of [2-^{3}H]-glycerol solution (250 μCi) were injected within about 5 sec into the femoral vein of the rats under slight ether anesthesia. After different time intervals, the chest was opened in 15 sec before the desired time period, and the lungs were quickly removed and frozen in liquid nitrogen. The time from the end of the injection to the beginning of the freezing was measured. The lung weight was 1.35 ± 0.37 g ($n=13$). The lung lipids were extracted from the frozen tissues with chloroform-methanol (2: 1, v/v) mixture. After washing the extracts with 0.9% saline, the lower phase was evaporated under nitrogen gas, redisolved in chloroform, and when necessary, was stored at –20°. The injection of [2-^{3}H]-glycerol was carried out in the same manner as described above. The efficiency of

the lipid extraction was checked by repeating the extraction procedure. The second extracts usually contained less than 3% of radioactivity obtained in the first extracts.

Fractionation of lipids

The lung lipids, together with 2 μmoles of cold phosphatidic acids, were first fractionated by DEAE-cellulose column chromatography by the method of Rouser et al. (1965) as described in detail by Akino et al. (1970). More than 90% of radioactivity and lipid-phosphorus applied to the column could be recovered in this chromatographic procedure. The neutral lipid fraction obtained from the column was further fractionated on thin-layer plates of Kiesel gel G with the solvent system of hexane-diethyl ether- acetic acid-methanol (90:20:2:3, by vol.), as described earlier (Kanoh and Ohno 1973b). 1,2-Diacylglycerols obtained by this procedure were found to be considerably contaminated with cholesterol, but this did not affect the analysis in this work. Fractionation of non-acidic phospholipids was achieved by thin-layer chromatography on Kiesel gel G with the system of chloroform-methanol-water (70:30:5, by vol.) (Kanoh 1969; Akino et al. 1970). The eluate from the column containing phosphatidic acids was washed repeatedly with 50% methanolic 0.9% NaCl solution to remove ammonium acetate, and then phosphatidic acids were purified by thin-layer chromatography on Kiesel gel H by the method of Skipski et al. (1967).

Subfractionation of lecithins, 1,2-diacylglycerols and phosphatidic acids according to the degree of unsaturation

The glycerolipids were converted to diacylglycerol acetates prior to the subfractionation, with non-radioactive carriers, when necessary. Approximately 3 μmoles of 1,2-diacylglycerols or lecithins, after being hydrolyzed with phospholipase C, were acetylated with 2 ml of acetic anhydride and 0.4 ml of pyridine at 20° for 20 hr, as described by Renkonen (1966). Phosphatidic acids could be converted to diacylglycerol acetates by acetolysis by Renkonen's method (1965). In these procedures, a care was taken to handle 1,2-diacylglycerols as rapidly as possible to avoid possible isomerization of the compounds, and no significant formation of 1,3-isomer was detected on thin-layer chromatography. 1,2-Diacylglycerol acetates thus obtained were subfractionated by thin-layer chromatography on Kiesel gel G impregnated with 10% $AgNO_3$ by slightly modified method of Hill et al. (1968). The plates were first developed with ether-hexane-benzene (60:30:2.5, by vol.) to the height of 10 cm from the origin. After drying the plates under nitrogen flow, the second development was fully performed with hexane-diethyl ether-benzene-methanol (65:10:25:0.5, by vol.) system. After detecting the bands with dichlorofluorescein, the fractionated species were eluted with chloroform-methanol (4:1, v/v) and were washed successively with 1 M NH_4OH and 50% methanolic 0.9% saline. By this procedure, six major species of glycerolipds, i.e., saturated, mono-, di-, tri-, tetra- and hexaenoic species, could be clearly separated. The efficiency of the fractionation was confirmed by gas-liquid chromatography (Kanoh 1969; Akino et al. 1970).

Determination of positional distribution of radioactivity in glycerolipids

Distribution of radioactivity in the 1- and 2-positions of unfractionated lecithins and 1,2-diacylglycerols, after being converted to phosphatidylphenols by Åkesson's method (1969), was determined by phospholipase A (*Crotalus adamanteus*) hydrolysis, as described by Åkesson (1969) and Kanoh (1969). Intact lecithins were first subfractionated to separate, as much as possible, diplamitoyl lecithin from other unsaturated species (Kanoh 1969; Akino et al. 1971). Dipalmitoyl lecithin thus fractionated was found to be still contaminated with monoenoic species and was further degraded by permanganate-periodate oxidation (Shimojo T., paper in preparation) to remove contaminating unsaturated species. Dipalmitoyl lecithin and other unsaturated species could be analyzed by snake venom treatment.

Saturated and unsaturated species of 1,2-diacylglycerols were also analyzed. In this case, subfractionated 1, 2-diacylglycerol acetates derived from 1, 2-diacylglycerols were

hydrolyzed by hog pancreatic lipase by the method of Luddy et al. (1964). 5 μmoles of 1,2-diacylglycerol acetates were incubated with 0.05 mg of lipase for 10 min at 37°. Under this condition, 14.5–20% of the incubated 1,2-diacylglycerol acetates could be recovered as monoacylglycerol. From the specific radioactivity of the original diacylglycerol acetates and the formed monoacylglycerol, it became possible to determine the positional distribution of radioactivity in each species of 1,2-diacylglycerols.

Disaturated 1,2-diacylglycerol could also be obtained by the oxidation of phsphatidylphenols derived from total 1,2-diacylglycerols and was analyzed by phospholipase A degradation. Both methods gave similar results.

Analytical methods

Phosphorus was measured by Bartlett's method (1959). The amounts of 1,2-diacylglycerols and 1,2-diacylglycerol acetates were determined by the method of Van Handel and Zilversmit (1957). In most cases radioactivity was determined in Bray's scintillation fluid (Bray 1960), using a liquid scintillation counter model LS-500, Horiba, Japan. In thin-layer chromatography, the spots were localized by slight exposure to iodine vapor, directly scraped off into counting vials, and radioactivity was measured in dioxane-water system (Synder 1964).

Recovery of radioactivity was checked with known amounts of standard materials labeled with ^3H. It was found that the recovery of radioactivity of lysophosphatidylphenols, $73.7 \pm 4.3\%$ (n=5) and of lysolecithin, $75.2 \pm 1.3\%$ (n=5) was significantly lower than that of fatty acids, $88.2 \pm 2.2\%$ (n=5). This difference of the recovery was corrected to determine the positional distribution of radioactivity in the glycerolipids (See the pevious section).

Others

After injection of [9,10-$^3H_2$]-palmitic acid, fatty acid methyl esters were prepared from the extracted lung lipids in each period and were analyzed by argentation thin-layer chromatography as described previously by Kanoh and Lindsay (1972). Usually over 95% of the radioactivity was found to migrate with methyl esters of saturated acid, indicating that the conversion of injected palmitic acid to unsaturated acids could be considered negligible in the present experimental conditions.

Calculation of turnover time, turnover rate and turnover rate constant of molecular species of rat lung lecithins in de novo synthesis

From the time-dependent change of specific radioactivity of each molecular species of 1,2-diacylglycerols and lecithins, the turnover time and turnover rate of lung lecithins could be calculated according to Zilversmit et al. (1943) and Wise and Elwyn (1965), as described in details by Sakamoto and Akino (1972) and Toshima et al. (1972). The pool size of 1,2-diacylglycerols was determined in this work and the data on lecithin contents in rat lung were taken from the previous report by Akino et al. (1971). In these calculations, it was assumed that the pool of rat lung glycerolipids was homogeneous and remained constant during the experiments and that there was no significant effect of other pathways than synthesis de novo on the formation of lecithins during the period of 2–10 min.

Results

The tissue content of 1,2-diacylglycerols

The tissue content of 1,2-diacylglycerols, the direct precursors for de novo synthesis of lecithins, has not yet been reported in rat lung, and was analyzed in the present investigation in order to obtain quantitative data. The results are given in Table 1. The pool size of 1,2-diacylglycerols obtained in this work was found to be similar with that reported by Åkesson (1969) in rat liver, but the

analysis of molecular species showed that rat lung 1,2-diacylglycerols are the mixture of fairly equal amounts of fatty acid species, being significantly different from the reported composition of these compounds in rat liver (Åkesson 1969). Much less content of saturated 1,2-diacylglycerol was noted, when compared to the

TABLE 1. *Analysis of 1,2-diacylglycerols in rat lung*

Tissue content (μmole/g wet tissue)	0.38±0.05(5)
Molecular composition	mole %
Saturated	10.6±2.4
Mono-	24.2±6.1
Di-	19.7±3.0
Tri-	10.9±3.4
Tetra-	19.9±3.9
Hexaenoic	14.6±8.9

The lung tissues collected from 5 to 10 animals were analyzed in each determination. Total lipid extracts obtained from the tissues were fractionated on thin-layer plates and 1,2-diacylglycerols were quantitatively recovered. Subfractionation of the 1,2-diacylglycerols was performed by argentation thin-layer chromatography after being converted to diacylglycerol acetates. The data are given as average values±S.D. with the number of independent determinations in the parenthesis.

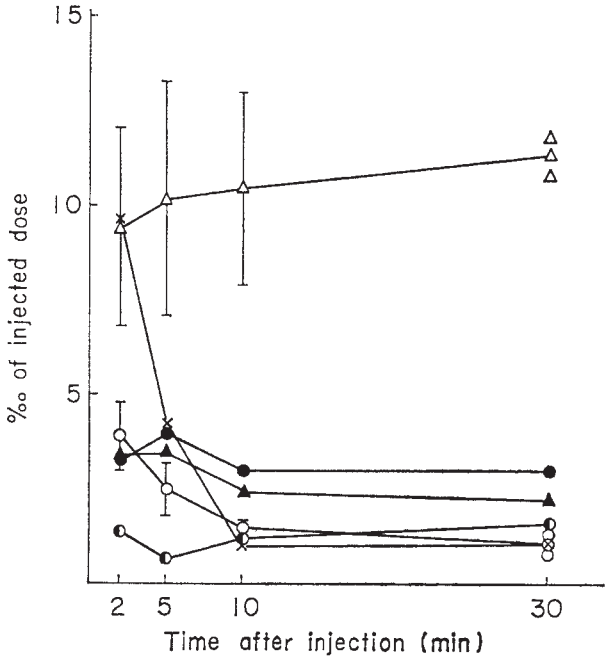

Fig. 1. Incorporation of [9,10-$^3H_2$]-palmitic acid into rat lung lipids after intrafemoral injection.

Lipids were fractionated by DEAE-cellulose column and thin-layer chromatography and radioactivity in each lipid class was obtained. For lecithins and 1,2-diacylglycerols, the data are given as the mean values ±S.D. from 3 independent experiments. The range of deviation was omitted in other fractions for technical reasons and the averages from 2 to 3 animals are presented.

△—△, lecithins; ○—○, 1,2-diacylglycerols; ×—×, fatty acids; ◦—◦, phosphatidylethanolamines; •—•, triacylglycerols and ▲—▲, other phospholipids.

very high amount of dipalmitoyl lecithin in rat lung reported previously by Akino et al. (1971).

Experiment with [9,10-$^3H_2$]-palmitic acid

Fig. 1 shows the appearance of the injected [9,10-$^3H_2$]-palmitic acid in the rat lung lipids. 2.5±0.7% (10) of the injected radioactivity was incorporated into lung lipids and this percentage remained fairly constant during the experimental periods. The radioactivity in fatty acids rapidly disappeared after 2 min and a gradual increase of radioactivity in lecithins was accompanied by a corresponding decrease of 1,2-diacylglycerols, suggesting that the formation of lecithins during the period of 2–30 min was mainly due to the de novo synthesis mechanism through 1,2-diacylglycerols. Only 0.73±0.12% (3) of the incorporated radioactivity was found in phosphatidic acid in 2 min and the analysis of this compound could be performed only partially. Time course of the change of specific radioactivity of the lung lipids is presented in Fig. 2. Specific radioactivity of 1,2-diacylglycerols was found to be much higher than that of lecithins throughout the experimental periods, confirming the suggestion that the lecithins synthesized

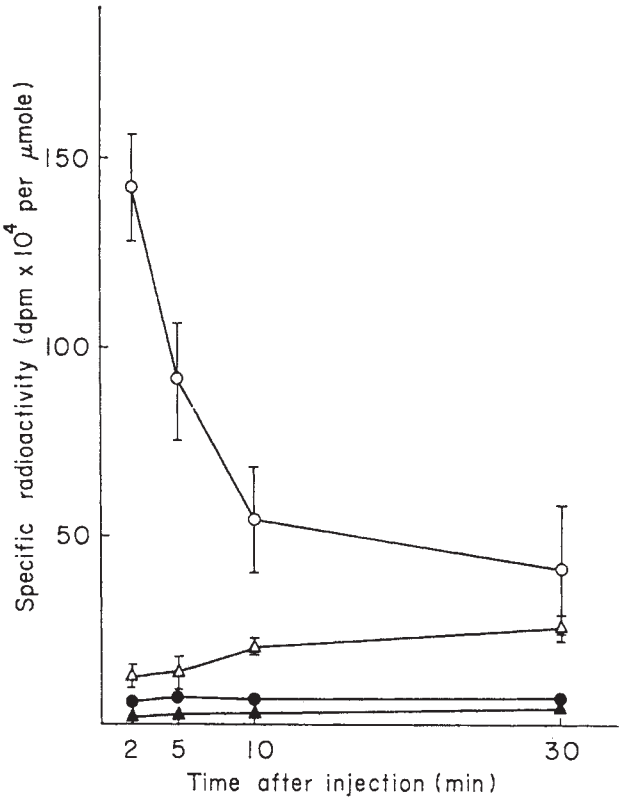

Fig. 2. Changes of specific radioactivity of rat lung glycerolipids after intrafemoral injection of [9,10-$^3H_2$]-palmitic acid.
Each lipid was purified by thin-layer chromatography and specific radioactivity was determined in the periods indicated. The average values obtained with 3–4 animals are given with the range of S.D. The range of deviation was omitted in triacylglycerols and phosphatidylethanolamines for technical reasons. o—o, 1,2-diacylglycerols; △—△, lecithins; ▲—▲, phosphatidylethanolamines and •—•, triacylglycerols.

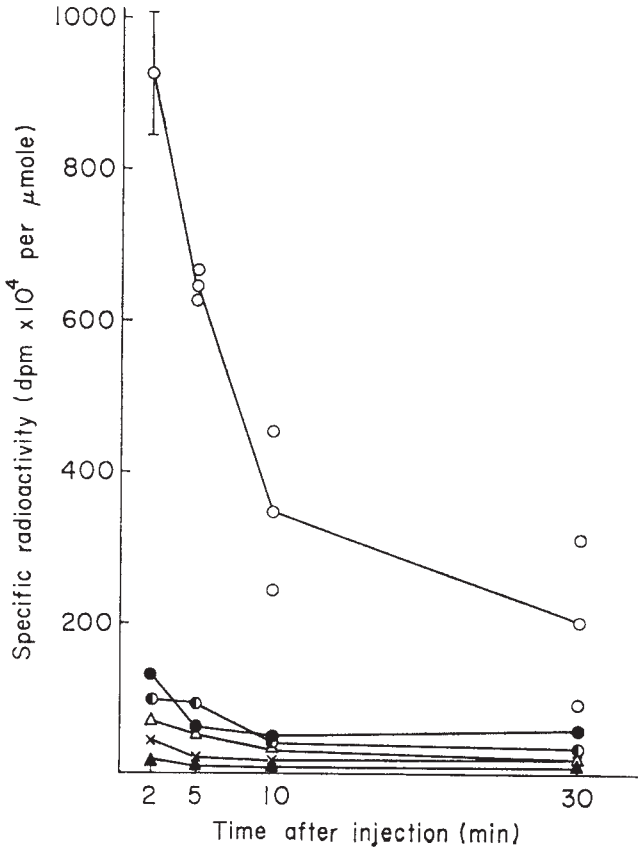

Fig. 3. Time-dependent change of specific radioactivity of the molecular species of rat lung 1,2-diacylglycerols after injection of [^{3}H]-palmitic acid.
Specific radioactivity of each 1,2-diacylglycerol species was calculated from specific radioactivity of the unfractionated 1,2-diacylglycerols and from the data given in Tables 1 and 2. Each mark is the average value from 2–3 animals.
○—○, saturated; ◐—◐, mono-; △—△, di-; ×—×, tri-; ▲—▲, tetra- and ●—●, hexaenoic species of 1,2-diacylglycerols.

TABLE 2. *Distribution of radioactivity in molecular species of glycerolipids of rat lung after injection of [9,10-$^3H_2$]-palmitic acid*

Lipid class	Time after injection (min)	Distribution of radioactivity in molecular species (%)					
		Saturated	Mono-	Di-	Tri-	Tetra-	Hexaenoic
Phosphatidic acids	2(3)	60.2±1.0	23.4±2.2	7.9±2.3	3.5±0.5	0.9±0.1	4.1±0.4
1,2-Diacylglycerols	2(4)	56.8±3.2	19.8±4.1	10.5±0.5	3.3±1.0	2.2±1.3	7.4±2.9
	5*	55.4	25.1	10.6	2.0	2.1	4.8
	10*	53.2	21.8	12.5	2.4	3.4	6.7
	30*	40.4	20.9	13.0	6.0	5.7	14.0
Lecithins	2(4)	60.9±3.7	16.6±1.6	10.3±1.5	3.6±0.7	3.0±1.2	5.6±1.2
	5(4)	61.8±4.4	16.1±0.9	10.9±1.9	3.3±0.9	3.0±1.3	4.9±1.3
	10(3)	58.3±1.7	17.1±1.7	12.5±0.8	3.1±1.1	3.3±0.2	5.7±1.2
	30*	57.0	18.8	12.4	3.3	4.1	4.5

Glycerolipids of the rat lung were analyzed at the time indicated after intrafemoral injection of 100 μCi of [9,10-$^3H_2$]-palmitic acid.
* An average value from 2 animals is presented. Other details are the same as described in Table 1.

during the period of 2–30 min should be mainly due to the action of 1,2-diacylglycerol: CDP-choline cholinephosphotransferase. Fig. 3 shows the change of specific radioactivity of molecular species of 1,2-diacylglycerols, which could be calculated from the specific radioactivity of unfractionated 1,2-diacylglycerols and from the data presented in Table 1. The saturated species of 1,2-diacylglycerols was found to have the highest specific radioactivity and to change most rapidly during the experiments. When the labeling of the molecular species of lecithins was studied (see Fig. 4), dipalmitoyl lecithin was observed to have the highest specific radioactivity, but it should be mentioned that the rate of the increase of the specific radioactivity was not so different among the molecular species of rat lung lecithins. Distribution of radioactivity among the subfractions of glycerolipids was studied and the data are given in Table 2. The results showed that the mode of the incorporation of palmitic acid into phosphatidic acids and 1,2-diacylglycerols was quite different from that observed in the similar experiments in rat liver (Åkesson et al. 1970b), forming actively disaturated species. It seems that this labeling pattern of the precursors of the synthesis de novo could be reflected on the formation of lecithin species. Vereyken et al. (1972) previously studied the incorporation of injected [2-^{3}H]-glycerol into rat lung lecithins, and they did not find the marked formation of dipalmitoyl lecithin. To clarify this discrepancy we injected [2-^{3}H]-glycerol and studied in more details the formation of molecular species of rat lung glycerolipids in the next experiments.

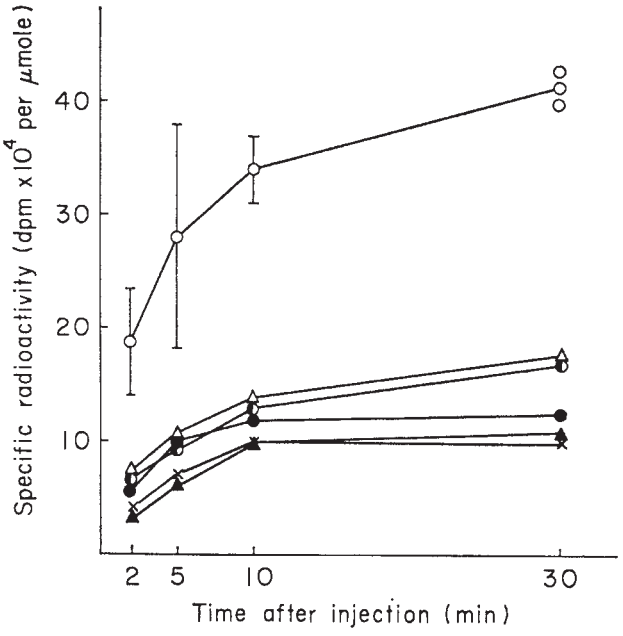

Fig. 4. Time-dependent change of specific radioactivity of the molecular species of rat lung lecithins.
Specific radioactivity of lecithin species was obtained by argentation thin-layer chromatography in the indicated periods after injection of [^{3}H]-palmitic acid. Each point represents the average values from 2–4 animals.
○—○, dipalmitoyl; ◐—◐, mono-; △—△, di; ×—×, tri-; ▲—▲, tetra- and ●—●, hexaenoic species of lecithins.

Experiments with [2-^3H] glycerol

Rat lung lipids were analyzed in 5 min after injection of [2-^{3}H]-glycerol, and the results are presented in Table 3. Saturated species of 1,2-diacylglycerols was again found to be most active in taking up glycerol, confirming the results obtained with [9,10-$^3H_2$]-palmitic acid. However, the formation of dipalmitoyl lecithin was not found to be so marked as that expected from the data given in Table 2. The highest specific radioactivity was found in di- and trienoic species in agreement with the results by Vereyken et al. (1972) and the percentage of the formed dipalmitoyl lecithin could not explain the very active formation of this species observed in the experiments with [9,10-$^3H_2$]-palmitic acid (See Table 2), as the results with [2-^{3}H]-glycerol could be considered to represent the relative amounts of palmitic acid incorporated into the 1-position. We therefore determined the distribution of radioactivity in the 1- and 2-positions of glycerolipids formed from [9,10-$^3H_2$]-palmitic acid.

TABLE 3. *Incorporation of [2-^3H]-glycerol into 1,2-diacylglycerols and lecithins in 5 min after intrafemoral injection*

Lipid class	Molecular species	Distribution of radioactivity (%)	Specific radioactivity ($\times 10^4$ dpm/μmole)
Diglycerides (3)	Saturated	33.0±0.5	83.8
	Mono-	26.8±2.7	29.9
	Di-	19.8±3.2	28.3
	Tri-	8.3±1.9	17.5
	Tetra-	2.7±0.4	3.8
	Hexaenoic	9.4±2.8	19.1
Lecithins (4)	Saturated	25.8±3.7	2.4±0.3
	Mono-	22.3±2.8	2.5±0.5
	Di-	28.2±3.9	4.9±1.3
	Tri-	10.8±2.7	5.4±2.4
	Tetra-	4.1±0.1	1.2±0.3
	Hexaenoic	8.8±1.6	3.0±1.0

Formation of molecular species of 1,2-diacylglycerols and lecithins was studied in 5 min after injection of [2-^{3}H]-glycerol. Specific radioactivity of 1,2-diacylglycerols is the average of two experiments.
See Table 1. for other details.

Positional distribution of radioactivity in rat lung glycerolipids

The distribution of radioactivity between 1- and 2-positions of the total and subfractionated 1,2-diacylglycerols and lecithins was determined and the results are given in Table 4. It can be seen that the radioactivity incorporated into the 2-position of the total lecithins was found to be abnormally high and about 60% of the radioactivity was located in the 2-position. The same results were already noted by Akino et al. (1971) in the experiments with rat lung slices. Also the ratio of distribution of radioactivity between the 1- and 2-positions of dipalmitoyl lecithin was approximately 1:3 on the average during the experimental periods, although a relatively low ratio was found in 30 min. It was also noted that the

TABLE 4. *The positional distribution of radioactivity in rat lung glycerolipids after injection of [9,10$^3H_2$]-palmitic acid*

Time after injection (min)	Total 1,2-diacylglycerol	Saturated 1,2-diacylglycerol	Unsaturated 1,2-diacylglycerol	Total lecithin	Dipalmitoyl lecithin	Unsaturated lecithin
2	42.1±1.0(4)	52.8	27.1	59.5±2.3 (3)	76.4	26.1
5	42.6	49.7	n.d.	53.9±2.6 (3)	82.1	15.5
10	49.1	48.6	n.d.	54.5	73.0	22.6
30	44.1	48.5	n.d.	49.5	69.7	21.6

Radioactivity found in the 2-position (%)
Positional distribution of radioactivity was analyzed in unfractionated and subfractionated 1,2-diacylglycerols and lecithins after injection of [9,10$^3H_2$]-palmitic acid by phospholipase A or pancreatic lipase treatment.
The values, given without S.D., are the averages of two independent determinations with similar results.
n.d.: not determined.

unsaturated species of rat lung lecithins took up palmitic acid considerably into the 2-position.

The data obtained in 5 min in saturated and unsaturated lecithins were taken to calculate the positional distribution of radioactivity in unfractionated lecithins as well as the relative amounts of radioactive palmitic acid incorporated into the 1-position, which could be regarded as being comparable to the data obtained with [2-^{3}H]-glycerol (See Table 3). In this calculation the results presented in Table 2 were employed. The calculated radioactivity in the 2-position of unfractionated lecithins was found to be 51.3% (actually found: 53.9%, see Table 4), and the relative amount of the formed dipalmitoyl lecithin was calculated to be 25.6%; these values are closely consistent with the results presented in Table 3. When the same analysis was performed in 1,2-diacylglycerols, the ratio of the distribution of radioactivity in the two positions of saturated species was found to be almost 1:1 and a considerable radioactivity was located in the 2-position of unsaturated species (see Table 4). Although the analysis of unsaturated 1,2-diacylglycerol species could be carried out only in 2 min, because of the inadequate amounts of radioactivity, the data given in Table 4 could reasonably explain the results obtained with radioactive glycerol (see Table 3). It now seems possible that in rat lung, intrafemorally injected palmitic acid is incorporated into the 2-position of dipalmitoyl lecithin by other mechanism than de novo synthesis without being thoroughly mixed with the endogenous intracellular fatty acid pool. This unknown mechanism might function much earlier than 2 min after injection of the precursor and could be still observed in spite of the inflow of radioactivity from 1,2-diacylglycerols in the present experimental periods. In this context it should be noted that within as short as 2 min after the injection of radioactive precursor a much higher radioactivity was incorporated in lecithins than that incorporated during the period between 2 to 30 min of the experiment and that the observed rapid

decrease of radioactivity in fatty acid suggests an almost pulse-labeling of the lung lipids by the injected palmitic acid (see Fig. 1).

Calculation of turnover time, turnover rate and turnover rate constant of the molecular species of lecithins in de novo synthesis

The time-dependent change of specific radioactivity of 1,2-diacylglycerols and lecithins, already presented in Figs. 3 and 4, respectively, has been summarized in Fig. 5 in order to compare the changes between the same molecular species of the two compounds. As seen in Fig. 5, the behavior of tetraenoic lecithin was

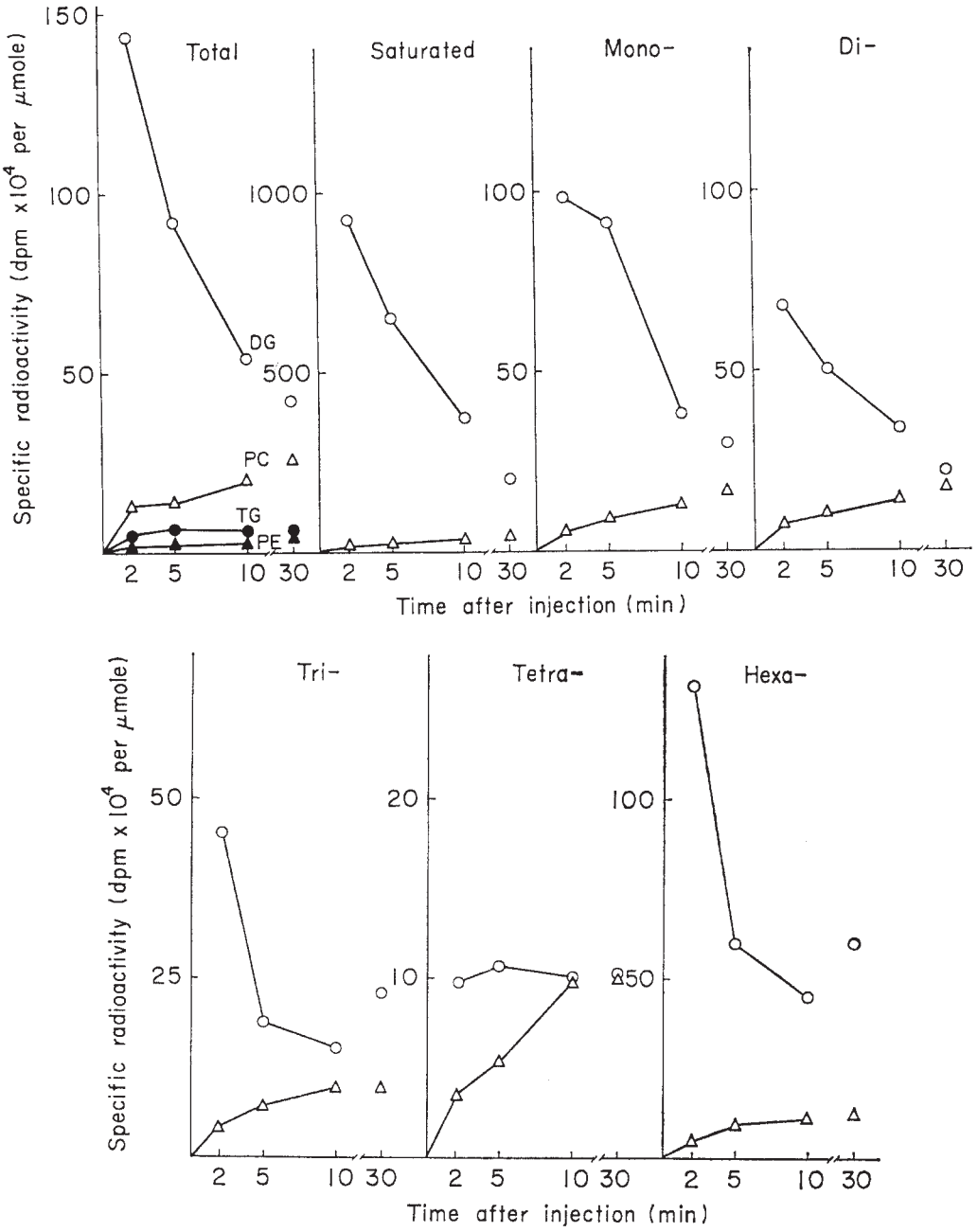

Fig. 5. Time-dependent change of corresponding molecular species of 1,2-diacylgycerols and lecithins after injection of [^{3}H]-palmitic acid.
See Figs. 3 and 4 for details.

found to be abnormal, rapidly equilibrating with 1,2-diacylglycerol. This abnormal behavior of tetraenoic lecithin will be discussed in detail in the section of Discussion. Either the effects of reacylation mechanism of 1-acyllysolecithin (Kanoh 1969, 1970; Akino et al. 1971, 1972; Kanoh and Ohno 1973 a, b;) or the influence of the reversibility of 1,2-diacylglycerol: CDP choline cholinephosphotransferase (Kanoh and Ohno 1973 a, b) may be responsible for this phenomenon. From the data given in Fig. 5, it became possible to calculate the turnover time of each molecular species of lecithins. The turnover rate could also be readily calculated from the observed turnover time by using the data of the pool size of rat lung lecithins reported previously by Akino et al. (1971). The turnover rate constants could be estimated from the turnover rates obtained and from the tissue contents of 1,2-diacylglycerols presented in Table 1. The details of the calculations have been described in Method. As shown in the previous sections of the present paper, the positional distribution of radioactivity in dipalmitoyl lecithin was confirmed to be different from that in saturated 1,2-diacylglycerol, but this difference did not affect the present calculation, as the changes of specific radioactivity from 2 to 10 min were taken into consideration. The results of the calculations are given in Table 5. The turnover rate constant of lecithin species did not show marked differences except a low value obtained with hexaenoic species. The abnormally high values observed in tetraenoic lecithin could not be accepted as such from the reasons described above. The calculated turnover rate showed that no more than 17% of lecithins formed by de novo synthesis mechanism was of dipalmitoyl type, in spite of the marked formation of disatuated species of phosphatidic acid and 1,2-diacylglycerols observed in the present work. These results may suggest that the de novo synthesis pathway in rat lung cannot be regarded as being fully responsible for the formation of dipalmitoyl lecithin, in view of the very high content of this specific lecithin in this tissue (Akino et al. 1971). The relative amount of dipalmitoyl lecithin formed

TABLE 5. *The results of calculations of turnover time, turnover rate and turnover rate constant of rat lung lecithins synthesized de novo*

Subfractions of lecithins	Turnover time (min)	Turnover rate (μmoles/g tissue/min)	Turnover rate constant (min^{-1})
Total	90	0.106	0.28
Dipalmitoyl	200	0.017	0.42
Mono-	90	0.027	0.30
Di-	45	0.040	0.60
Tri-	20	0.015	0.38
Tetra*-	8*	0.137*	1.8*
Hexaenoic	90	0.005	0.09

The time-dependent change of specific radioactivity of each molecular species of 1,2-diacylglycerols and lecithins from 2 to 10 min was used for the calculation. (See Fig. 5)

* Extremely rapid turn over calculated in tetraenoic lecithin should be due to the rapid equilibration of this species with tetraenoic 1,2-diacylglycerol, as shown in Fig. 5.

from [2-^{3}H] glycerol was observed to be apparently lower than that of disaturated 1,2-diacylglycerol (See Table 3). This discrepancy may also be explained with the relatively lower turnover rate calculated in dipalmitoyl lecithin.

Discussion

From the results obtained with [^{3}H-]palmitic acid and [^{3}H]-glycerol, the present work showed that the formation of phosphatidic acids and 1,2-diacylglycerols in rat lung is markedly different from that observed in rat liver (Hill et al. 1969; Åkesson et al. 1970 a, b), and the disaturated species was found to be most active to take up the radioactive precursors. It seems that acyl-coenzyme A: 1-acylglycerophosphate acyltransferase may utilize preferentially palmitic acid, while the transferase has been shown to be relatively specific towards oleic and linoleic acid in rat liver microsomes (Okuyama and Lands 1972). Detailed studies in the cell-free systems will be necessary to confirm the observed difference of phosphatidic acid-synthesizing system between the lung and liver tissues. In spite of this preferential formation of disaturated 1,2-diacylglycerol, we could not find marked differences in the calculated turnover rate constants of lecithins synthesized de novo, as far as dipalmitoyl, monoenoic, dienoic and trienoic species were concerned. This result may indicate the same non-selectivity of the action of 1,2-diacylglycerol: CDP-choline cholinephosphotransferase in utilizing 1,2-diacylglycerol species, as reported in detail by Kanoh (1970) and Kanoh and Ohno (1973a, b) in rat liver microsomes. The calculated turnover rate showed that dipalmitoyl species comprised only about 17% of lecithin formed by the de novo synthesis mechanism in this work. This result may explain the discrepancy between the formations of disaturated species of 1,2-diacylglycerols and lecithins, observed in the experiments with [^{3}H]-glycerol (see Table 3) and may also suggest that, although dipalmitoyl lecithin can be formed partially by the pathway of synthesis de novo, this biosynthetic route cannot be regarded as being fully responsible for the formation of this specific lecithin in rat lung, in view of the very high content of this lecithin species as reported in this tissue by Montfoort et al. (1971) and Akino et al. (1971). From the analysis of the positional distribution of radioactivity in molecular species of glycerolipids and also from the comparison of the results between [^{3}H]-palmitic acid and [^{3}H]-glycerol injection, it was shown that palmitic acid of a higher specific radioactivity was incorporated into the 2-position of dipalmitoyl lecithin in the earliest period after administration of [^{3}H]-palmitic acid.

The three biosynthetic routes, so far reported as being related to the synthesis of dipalmitoyl lecithin in the lung cannot be considered to explain the above finding. Reacylation mechanism of lysolecithin (Frosolono et al. 1971; Vereyken et al. 1972) cannot be considered to cause the above effects, as the microsomal fraction obtained from the lung tissue has been shown to reacylate equally both 1- and 2-positions of lysolecithin (Frosolono et al. 1971). The transfer of palmitic acid between two molecules of 1-palmitoyllysolecithin (Akino et al. 1971; Abe et

al. 1972, 1973; Abe and Akino 1972) could not concentrate the radioactivity of [^{3}H]-palmitic acid in the 2-position, and the contribution of the de novo synthesis pathway seems inplausible, as no corresponding phenomenon could be detected in the precursor, disaturated 1,2-diacylglycerol. The mechanism of this unusual phenomenon remains to be seen, but the presence of acyl-coenzyme A: lysolecithin acyltransferase in the plasma membrane of lymphocytes (Ferber and Resch 1973) as well as of erythrocytes (Waku and Lands 1968) may be of some help to understand the above findings.

The time-dependent change of tetraenoic lecithin was found to be quite abnormal, as given in Fig. 4, and a rapid equilibration between tetraenoic species of 1,2-diacylglycerols and lecithins was clearly observed. Reversibility of the action of 1,2-diacylglycerol: CDP choline cholinephosphotransferase has been shown in rat liver in the experiments in vivo (Bjørnstad and Bremer 1966) as well as in vitro (Kanoh and Ohno 1973 a, b). If the same reversibility of cholinephosphotransferase could be assumed in rat lung, the effects of the back reaction of the transferase will appear most rapidly in tetraenoic species of 1,2-diacylglycerols and lecithins, as both compounds have been found to be most inactive to incorporate the radioactive precursors (see Figs. 3 and 4, Table 2 and 3).

Trienoic species was also found to be inactive to take up [^{3}H]-palmitic acid as shown in Figs. 3 and 4, but this species did not show abnormal behaviors. This may be due to the very low amount of this species in lung lecithins (Akino et al. 1971). Tetraenoic lecithin has been confirmed to be mainly synthesized through reacylation mechanism in rat liver (Kanoh 1969), and later the same results have been reported in various tissues including lung by Akino et al. (1972). Conversion of once-formed oligoenoic species into tetraenoic lecithin may also affect the behavior of this species in the present investigation.

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