REVISTA ESPAÑOLA DE FISIOLOGIA, 37, 477-484. 1981

Deacylation and Reacylation of Phosphatidylcholines by Liver Homogenates from Chick Embryos

J. Coll * and A. Ribera

Departamento de Bioquímica
Universidad Complutense
Madrid - 3 (Spain)

(Received on March 6, 1981)

J. COLL and A. RIBERA. *Deacylation and Reacylation of Phosphatidylcholines by Liver Homogenates from Chick Embryos.* Rev. esp. Fisiol., **37**, 477-484. 1981.

Liver homogenates from 13, 17 and 21 day-old chick embryos were incubated with phosphatidylcholine and lysophosphatidylcholine labeled with fatty acids to study deacylation and reacylation reactions of phosphatidylcholines during development. By using phosphatidylcholines labeled with palmitic or linoleic acids in position 1 or 2, respectively, phospholipase A_2 activity was detected. By using lysophosphatidylcholine labeled with palmitic acid in position 1, preferential acylation with araquidonic acid was demonstrated. In agreement with this data, the incubation of dienoic phosphatidylcholines labeled with palmitic acid in position 1, with the liver homogenates, gave rise to labeled tetraeonic phosphatidylcholines. The extent of both deacylation and reacylation of the phosphatidylcholine molecule in position 2 by liver homogenates from chick embryos, decreased during development.

Earlier studies by others (1, 13) have described the specific pattern of composition in molecular species of phosphatidylcholine characteristic of developmental stages of the chick embryo liver. An increase in stearoyl/linoleoyl species and a decrease in palmitoyl/araquidonoyl species and a decrease in palmitoyl/araquidonoyl species occurs during development.

We have previously shown (4) that palmitoyl/linoleoyl and palmitoyl-araquidonoyl phosphatidylcholines are actively synthesized by liver homogenates from 13, 17 and 21 day-old chick embryos. In this paper we report studies on the deacylation-reacylation reactions which occur in liver homogenates from chick embryos.

Materials and Methods

Obtention of labeled phospholipids. Livers from 17 day-old chick embryos (Yaniv race) were pooled, Dounce-homogenized in 20 mM Tris HCl-125 mM KCl pH 7.4 (Tris-KCl buffer) and centrifuged

* Present address: Instituto Tecnológico para Postgraduados. Paseo de Juan XXIII, 3, Madrid - 3 (Spain).

To whom correspondance should be address.

at 700 × *g* 18 min. The supernatant was obtained and adjusted to 16 mg/ml of protein as determined by the method of LOWRY *et al.* (14). Radioactive palmitic or linoleic acids were dispersed in Tris-KCl buffer with an MSE sonicator prior to the incubation. Ten tubes, containing each 3 μCi of fatty acid and 8 mg of protein from the 17 day-old chick embryos livers in a final volume of 1 ml were incubated for 1 h at 37° C in a water bath with constant agitation. The reaction was stopped by the addition of 5 ml of chloroform/methanol (1:2). Lipids were extracted by the method of BLIGH and DYER (3) and the phosphatidylcholines were isolated by thin layer chromatography over silica gel G (Merck) using chloroform/methanol/water 65:25:4 (v/v/v) as developing system.

Palmitic acid labeled phosphatidylcholines were fractionated further by thin layer chromatography over silicagel impregnated with silver nitrate by the method of KYRIAKIDES and BALINT (10). Dienoic, tetraenoic and hexaenoic species were visualized under ultraviolet light after spreading the plate with 0.002 % dichlorofluorescein in methanol. Dienoic and tetraenoic species were separately eluted from the silica gel with 20 ml of methanol, concentrated with a rotaevaporator and rechromatographied over silica gel plates using chloroform/methanol/water 65:25:4 (v/v/v) in order to eliminate the silver nitrate. Phosphorous was determined by the method of BARLETT (2). Preparation of the methyl esters and analysis by gas liquid chromatography (in a Hewlett-Packard 5750 G gas chromatograph) were performed as described by METCALFE and SCHMIDT (12) and MADARIAGA *et al.* (11), respectively.

Palmitic acid labeled phosphatidylcholines were concentrated to dryness, redissolved in ether and digested with phospholipase A_2 from *Crotalus adamanteus* (Koch Light Lab.) as described by VAN GOLDE and VAN DEENEN (16). The reaction was stopped with methanol after 3 h of incubation at 29° C and concentrated to dryness. Lysophosphatidylcholine was isolated by thin layer chromatography using chloroform/methanol/water 65:25:4 (v/v/v) as solvent.

Radioactive fatty acids were from Radiochemical Centre: 1-^{14}C palmitic acid, sp. actv. 58 mCi/mmol; U-^{14}C palmitic acid, sp. actv. 237 mCi/mmol and 1-^{14}C linoleic acid, sp. actv. 58 mCi/mmol. Counting efficiency was 80 %.

Assays and analysis of results. Livers from 13, 17 and 21 day-old chick embryos were obtained, pooled and homogenized in Tri-KCl buffer as described before (4). Labeled phospholipids were concentrated to dryness, resuspended in Tris-KCl buffer and sonicated with an MSE sonicator for 1 min. Aliquots of the phospholipid dispersions were added to the incubation mixtures containing 8 mg of protein from the liver homogenates and Tris-KCl buffer in a total volume of 1 ml. The incubations were carried out in a shaking water bath at 37° C and the reactions were stopped by the addition of 5 ml of chloroform/methanol (1:2). Lipid extraction was by the method of BLIGH and DYER (3). The lipid extracts were fractionated by thin layer chromatography using chloroform/methanol/water 65:25:4 (v/v/v) as solvent. Following visualization of the lipid spots with iodine, the lipids were scraped into scintillation vials. To measure the radioactivity, 2 ml of scintillation liquid (4) were added to the vials containing the silicagel and the lipids. Then, vials were capped, agitated and counted in a Nuclear Chicago scintillation spectrometer, model 6766. Counting efficiency was 80 %.

Results

Liver phosphatidylcholines from 17 day-old chick embryos were labeled with palmitic acid (1-^{14}C or U-^{14}C) and with lin-

Table I. *Fatty acid composition of the phosphatidylcholines used as labeled precursors.* Labeled phosphatidylcholines were prepared by incubation of liver homogenates from 17 day-old chick embryos with palmitic or linoleic acids as described in methods. The labeled phosphatidylcholines were digested with phospholipase A_2 to prepare lysophosphatidylcholine or fractionated by thin layer chromatography on silver nitrate plates as described. Every lipid was converted into methyl esters and analysed by gas liquid chromatography. The fatty acids were identified by known standards. Composition is given in molar percentages.

Fatty acid	Molar percentages			
	Total	Lyso	Dienoic	Tetraenoic
Palmitic acid (16:0)	30	46	28	22
Stearic acid (18:0)	22	42	30	30
Oleic acid (18:1)	9	4	12	—
Linoleic acid (18:2)	14	5	30	—
Araquidonic acid (20:4)	20	—	—	48
Docosahexanoic acid (22:6)	4	—	—	—

oleic acid (1-^{14}C). Table I shows the fatty acid composition of total, lyso, dienoic and tetraenoic phosphatidylcholines used as labeled precursors.

Phosphatidylcholine and lysophosphatidylcholine from liver homogenates were isolated in three independent experiments and their average concentrations measured by phosphorous content. The endogenous pools were: 69.2, 94 and 80.6 nmoles of phosphatidylcholine/mg of protein and 0.31, 0.15 and 0.18 nmoles of lysophosphatidylcholine/mg of protein for 13, 17 and 21 day-old chick embryos, respectively.

To measure the deacylation of phosphatidylcholines by liver homogenates, palmitic and linoleic acid labeled phosphatidylcholines were added to the homogenate and incubated at 37° C. Table II shows that when palmitic acid labeled phosphatidylcholine was used the radioactivity (expressed in nmoles) appears in lysophosphatidylcholine whereas when linoleic acid labeled phosphatidylcholines were used' most of the radioactivity appears in the free fatty acid instead of lysophosphatidylcholine. No radioactivity was recuperated in any other lipid except in phosphatidylcholine. Apparently, liver homogenates from 13 day-old chick embryos were more active than from 17 or 21 day-old ones in liberating lysophosphatidylcholine from palmitic acid labeled phosphatidylcholines. The release of free fatty acid from linoleic acid labeled phos-

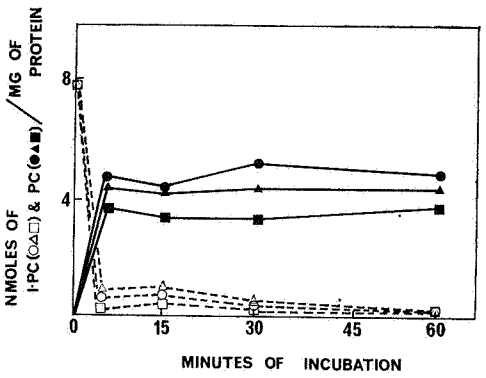

Fig. 1. *Time course of acylation of palmitic acid labeled lysophosphatidylcholine by liver homogenates from chick embryos.* Mixtures containing 62 nmoles of 1-^{14}C palmitic acid labeled lysophosphatidylcholine (specific activity: 0.4×10^6 dpm/μmol) and 8 mg of protein from liver homogenates in a final volumen of 1 ml, were incubated at 37° C for the periods of time indicated. Lipids were extracted and analysed as described in methods. Results are expressed in nmoles synthesized per mg of protein. Open symbols represent the nmoles of lysophosphatidylcholine. Closed symbols represent the nmoles synthesized of phosphatidylcholine. Liver homogenates were obtained from 13 (●, ○), 17 (▲, △) and 21 (■, □) day-old chick embryos.

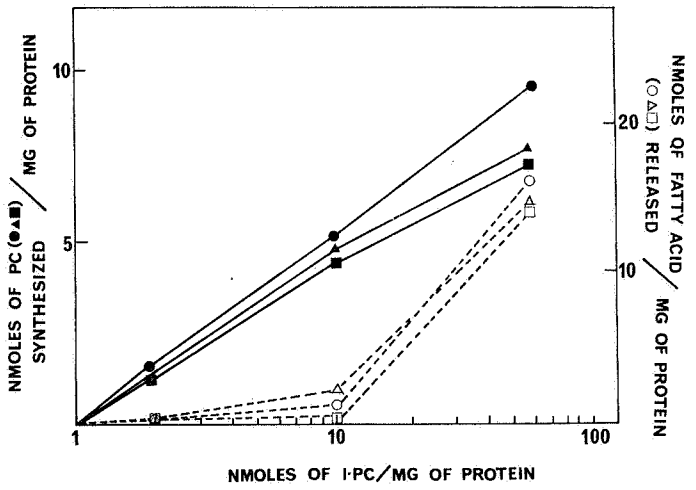

Fig. 2. *Effect of lysophosphatidylcholine concentration on synthesis of phosphatidylcholine and release of free fatty acids by liver homogenates from chick embryos.*
Mixtures containing different amounts of lysophosphatidylcholine (labeled with palmitic acid, specific activity: 1.1×10^6 dpm/μmol) and 8 mg of protein from the liver homogenates in a final volume of 1 ml were incubated at 37° C for 1 h. Lipids were extracted and analysed as described in methods. Results are expressed in nmoles/mg of protein. Closed symbols represent the nmoles of phosphatidylcholine synthesized. Open symbols represent the nmoles of free fatty acid released. Liver homogenates were obtained from 13 (●, ○), 17 (▲, △) and 21 (■, □) day-old chick embryos.

phatidylcholines was about equal for the three stages of development. This release was increasing with the time of incubation from 0 to 60 min at about the same rate for the 3 stages of development (data not shown).

Palmitic acid labeled lysophosphatidylcholines were incubated with liver homogenates for various periods of time. Figure 1 shows the phosphatidylcholine synthesized in this experiment increase rapidly and then plateaus after the first 5 min of incubation. This time course did not vary in other experiments using 500 nmoles of lysophosphatidylcholines (specific activity: 0.8×10^6 dpm/μmol). The synthesis of prosphatidylcholine from lysophosphatidylcholine was slightly greater in liver homogenates from 13 day-old chick embryos than in liver homogenates from 17 or 21 day-old chick embryos (fig. 1). This result did not vary by using different concentrations of lysophosphatidylcholine (fig. 2).

The synthesis of phosphatidylcholine increases linearly whereas the release of free fatty acids was high only when more than 10 nmoles of lysophosphatidylcholine/mg of protein were used (fig. 3). This effect was found in the 3 studied developmental stages.

The phosphatidylcholines resulting from the incubation with labeled lysophosphatidylcholine, were fractionated by thin layer chromatography over silica gel G impregnated with silver nitrate as described in methods. Figure 3 shows that tetraenoic species incorporated about 80 % of the total radioactivity recuperated in total phosphatidylcholines. The nmoles of tetraenoic phosphatidylcholine synthesized were greater in liver homogenates from

13 day-old chick embryos and decreased during development. The same specificity and variation throughout the development were found in experiments using different lysophosphatidylcholine concentrations.

Palmitic acid labeled phosphatidylcholines were fractionated by thin layer chromatography over silica gel impregnated with silver nitrate and dienoic and tetraenoic species were separately eluted, re-

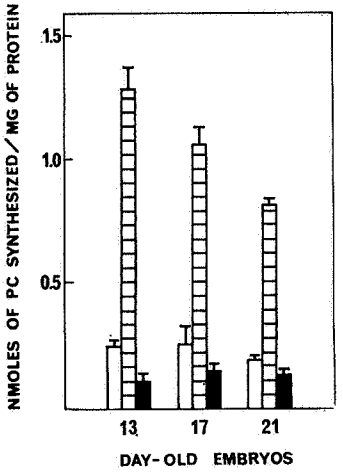

Fig. 3. *Incorporation of labeled lysophosphatidylcholine into fractionated phosphatidylcholines by liver homogenates from chick embryos.*
Mixtures containing 22 nmoles of lysophosphatidylcholine (labeled with palmitic acid, specific activity: 1.1×10^6 dpm/μmol) and 8 mg of protein from the liver homogenates in a final volume of 1 ml were incubated at 37° C for 1 hour. Phosphatidylcholines were isolated and fractionated by thin layer chromatography over silicagel impregnated with silver nitrate as described. Results are expressed as nmoles synthesized/mg of protein. The average value and the ranges from two independent experiments are given in the figure. Dienoic phosphatidylcholines (including saturated and monoenoic) are represented in white bars; tetraenoic phosphatidylcholines in hatched bars and hexaenoic phosphatidylcholines in black bars. Liver homogenates were obtained from 13, 17 and 21 day-old chick embryos.

Table II. *Deacylation of palmitic and linoleic acid labeled phosphatidylcholines by liver homogenates from chick embryos.*
Mixtures containing 25 nmoles of phosphatidylcholine (experiment A: labeled with palmitic acid, 85 % of the radioactivity in position 1; specific activity 1.7×10^6 dpm/μmol), 34 nmoles of phosphatidylcholine (experiment B: labeled with palmitic acid 85 % of the radioactivity in position 1; specific activity 1.98×10^6 dpm/μmol) or 88 nmoles of phosphatidylcholine (experiment C: labeled with linoleic acid, 90 % of the radioactivity in position 2; specific activity 1.98×10^6 dpm/μmol) and 8 mg of protein from liver homogenates in a final volume of 1 ml were incubated at 37° C for 5 min (exp. A), 15 min (exp. B) and 60 min (exp. C). The analysis of lipids was as described. The results are given in nmoles synthesized, calculated by taking into account the dilution with endogenous phosphatidylcholine. Experiment C was done in duplicate (averages and ranges).

Chick-embryo age	Experiment	nmoles/mg of protein	
		Free fatty acid	Lyso phosphatidylcholine
13 day-old	A	0.000	1.062
	B	0.053	3.900
	C	7.006 ± 0.1	1.200 ± 0.1
17 day-old	A	0.000	0.412
	B	0.035	0.350
	C	8.056 ± 0.5	0.180 ± 0.03
21 day-old	A	0.000	0.162
	B	0.000	0.000
	C	8.054 ± 0.4	0.018 ± 0.018

purified and incubated with liver homogenates from chick embryos. Figure 4 shows the time course of synthesis of tetraenoic species from dienoic species by liver homogenates. The synthesis increases during the first hour of incubation at different rates according with the age of the liver homogenate and decreases during development. No radioactivity could be found in the above experiments on the hexaenoic fractions. On the other hand, using labeled tetraenoic phosphatidylcholines (52 nmoles of U-^{14}C palmitic acid labeled, specific activity; 1.2×10^6 dpm/μmol) no radioactivity greater than back-

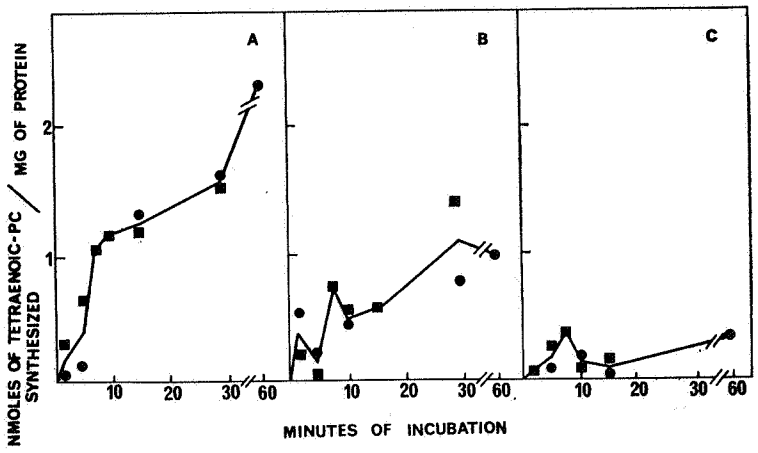

Fig. 4. *Time course of the transformation of dienoic into tetraenoic phosphatidylcholines by liver homogenates from 13 (A), 17 (B) and 21 (C) day-old chick embryos.* Mixtures containing 40 nmoles of dienoic phosphatidylcholines (experiment 1: labeled with 1-^{14}C palmitic acid, specific activity: $1{,}7 \times 10^6$ dpm/μmol) or 60 nmoles of dienoic phosphatidylcholines (labeled with U-^{14}C aplmitic acid, specific activity: 2.5×10^6 dpm/μmol) and 8 mg of protein from liver homogenates in a final volume of 1 ml were incubated at 37° C for the periods of time indicated. Phosphatidylcholines were isolated and fractionated as described. After background radioactivity was subtracted to every point (exp. 1: 400 cpm and exp. 2: 500 cpm), results were calculated in nmoles of tetraenoic phosphatidylcholines synthesized/mg of protein. Experiment 1 (●) and experiment 2 (■).

ground could be detected either in the dienoic or in hexaenoic fractions.

Discussion

Chick liver phosphatidylcholines specifically labeled with palmitic or linoleic acids have been used to study the presence of phospholipases in homogenates from chick embryo liver. Phospholipase A_2 activity could be demonstrated in liver homogenates from 13, 17 and 21 day-old chick liver embryos (table II). Phosphatidylcholine labeled with palmitic acid was hydrolyzed to lysophosphatidylcholine. The amount of lysophosphatidylcholine liberated decreased during development. Phosphatidylcholine labeled with linoleic acid was hydrolyzed to fatty acids. The amount of free fatty acids liberated remained constant during development.

Lysophosphatidylcholine labeled with palmitic acid was rapidly and specifically incorporated into tetraenoic phosphatidylcholine (figs. 1 and 3). Since the free fatty acids were produced only in the presence of more than 10 nmoles of lysophosphatidylcholine/mg of protein (fig. 2), the synthesis of tetraenoic phosphatidylcholine most likely occurs by direct acylation of lysophosphatidylcholine.

If reacylation of lysophosphatidylcholine occured when palmitic acid labeled phosphatidylcholines were incubated with liver homogenates (fig. 3 and table I), radioactivity should appear in the tetraenoic phosphatidylcholines. To test this hypothesis, dienoic phosphatidylcholines were incubated with liver homogenates and it was demonstrated that indeed tetraenoic

phosphatidylcholines were synthesized (fig. 4).

KANOH and AKESSON (9) measured phospholipase A_2 activity in cultured rat hepatocytes by pulse-chase experiments and demonstrated that arachidonate stimulated the synthesis of tetraenoic species suggesting the existence of a deacylation-acylation cycle. Many authors (5, 6, 8, 15, 18) had suggested the deacylation-acylation reactions to explain the high unsaturation degree of phosphatidylcholines in rat liver, on account of the specificity of the lysophosphatidylcholine acylation (7, 17). We report here a direct evidence for the existence of a deacylation-acylation cycle transforming dienoic into tetraenoic phosphatidylcholines in chick embryo liver.

The extent of phosphatidylcholine deacylation, lysophosphatidylcholine acylation and transformation of dienoic into tetraenoic phosphatidylcholines, decreases slowly during development. This decrease in the activity of the deacylation-reacylation cycle as measured *in vitro* correlates with the decrease in the proportion of tetraenoic phosphatidylcholines that occurs during development (1, 4, 13).

Resumen

Se estudian las reacciones de deacilación y reacilación durante el desarrollo hepático mediante homogenados de hígado de embriones de pollos de 13, 17 y 21 días incubados con fosfatidilcolina y lisofosfatidilcolina marcadas con ácidos grasos.

Utilizando fosfatidilcolinas marcadas con ácido palmítico o linoleico en posición 1 ó 2 respectivamente, se detecta actividad fosfolipasa A_2. Con lisofosfatidilcolina marcada con ácido palmítico en posición 1 se observa una acilación preferencial con ácido araquidónico. La incubación de fosfatidilcolina dienoica marcada en posición 1 con ácido palmítico da lugar a la aparición de fosfatidilcolina tetraenoica marcada. Tanto la deacilación como la reacilación de la posición 2 de la molécula de fosfatidilcolina disminuye durante el desarrollo del hígado de embrión de pollo.

References

1. ABAD, C., BOSCH, M. A., MUNICIO, A. M. and RIBERA, A.: *Biochim. Biophys. Acta*, **431**, 62-74, 1976.
2. BARLETT, G. R.: *J. Biol. Chem.*, **234**, 466-468, 1959.
3. BLIGH, E. G. and DYER, W. J.: *Canad. J. Biochem. Physiol.*, **39**, 911-917, 1959.
4. COLL, J. and RIBERA, A.: *Rev. esp. Fisiol.*, **37**, 469-476, 1981.
5. HILL, E. H. and LANDS, W. E. M.: *Biochim. Biophys. Acta*, **152**, 645-648, 1968.
6. HUSBANDS, D. R. and LANDS, W. E. M.: *Biochim. Biophys. Acta*, **202**, 129-140, 1970.
7. KANOH, H.: *Biochim. Biophys. Acta*, **176**, 756-763, 1969.
8. KANOH, H. and OHNO, K.: *Biochim. Biophys. Acta*, **380**, 199-207, 1975.
9. KANOH, H. and AKESSON, B.: *Biochim. Biophys. Acta*, **486**, 511-523, 1977.
10. KYRIAKIDES, E. C. and BALINT, J. A. J.: *Lipid. Res.*, **9**, 142-143, 1968.
11. MADARIAGA, A. M., MUNICIO, A. M. and RIBERA, A.: *Comp. Biochem. Physiol.*, **36**, 271-278, 1970.
12. METCALFE, L. E. and SCHMITZ, A. A.: *Anal. Chem.*, **33**, 363-364, 1961.
13. NOBLE, R. C. and MOORE, J. H.: *Can. J. Biochem.*, **45**, 627-639, 1967.
14. LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. and RANDALL, R. J.: *J. Biol. Chem.*, **193**, 265-275, 1951.
15. VAN DEN BOSCH, H., VAN GOLDE, L. M. G., EIBL, H. and VAN DEENEN, L. L. M.: *Biochim. Biophys. Acta*, **144**, 613-623, 1967.
16. VAN GOLDE, L. M. G. and VAN DEENEN, L. L. M.: *Chem. Phys. Lipids*, **1**, 157-164, 1967.
17. VAN GOLDE, L. M. G., PIETERSON, W. A. and VAN DEENEN, L. L. M.: *Biochim. Biophys. Acta*, **152**, 84-95, 1968.
18. VAN GOLDE, L. M. G., SHERPHOF, G. L. S. and VAN DEENEN, L. L. M.: *Biochim. Biophys. Acta*, **176**, 635-637, 1969.