REVISTA ESPAÑOLA DE FISIOLOGIA, 37, 469-476. 1981

Incorporation of Fatty Acids into Phosphatidylcholine Species 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. *Incorporation of Fatty Acids Into Phosphatidylcholine Species by Liver Homogenates from Chick Embryos.* Rev. esp. Fisiol., 37, 469-476. 1981.

Labeled palmitic, stearic, oleic, linoleic and araquidonic acids were incorporated into phosphatidylcholines by incubation with liver homogenates from 13, 17 and 21 day-old chick embryos. Palmitic acid was incorporated into dienoic and tetraenoic phosphatidylcholines whereas linoleic acid was incorporated into dienoic phosphatidylcholines. Labeled palmitic and linoleic acids were specifically found into positions 1 and 2 of the phosphatidylcholine molecule, respectively. Under *in vitro* conditions, the incorporation of either palmitic or linoleic acids into phosphatidylcholines was 2-3 fold greater in liver homogenates from 17 day-old chick embryos than in those from 13 or 21 day-old ones.

The variation in fatty acid composition (9, 10) and in individual molecular species (1) of phosphatidylcholine during the development of chick embryo liver has been reported. Both types of studies demonstrate about 60% decrease in the percentage of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine and a correspondent increase in the percentage of 1-stearoyl-2-lynoleoyl-sn-glycero-3-phosphocholine during the last week of embryonic development. Taking both phosphatidylcholine species together, they account for more than 30% of the total phosphatidylcholine species. Other phosphatidylcholines are either minor components or their proportions do not change during development (3). No attempts to explain these changes in terms of possible metabolic pathways, have been studied.

We report here, a cell-free system from embryonic chick liver to study the incorporation of fatty acids into phosphatidylcholines. The changes in the molecular species of phosphatidylcholine occuring

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

To whom correspondence should be address.

during development of chick embryo liver, can be partially explained by the *in vitro* utilization of labeled free fatty acids.

Materials and Methods

Incubation in vitro. Livers from 13, 17 and 21 day-old chick embryos were obtained from a commercially available source (Yaniv race). Several livers were pooled, homogeneized in 20 mM Tris-HCl, 125 mM KCl, pH 7.4 (Tris-KCl buffer) with a Dounce and centrifugated at 700 × *g* 10 minutes. The supernatants were adjusted to 16 mg/ml of protein as determined by the method of LOWRY *et al.* (11). All the steps were carried out at 0-5° C and the homogenates were used for the experiments within the next 2 hours. Radioactive fatty acids were dispersed in Tris-KCP buffer with an MSE sonicator and added to the incubation mixture containing 0.5 ml of liver homogenate from chick embryo and Tris-KCl buffer to a final volume of 1 ml. Incubation was 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) and lipids were extracted by the method of BLING and DYER (3). The lipid extracts were concentrated using a rotaevaporator and stored at —20° C in atmosphere of nitrogen.

Lipid analysis. The lipid residue was dissolved in chloroform-methanol (1:2) and aliquots were fractionated by thin layer chromatography over silicagel G (Merck) plates of 500 μ thick. Hexane-ether-acetic acid: 70:30:1 (v/v/v) was used to separate neutral lipids and chloroform-methanol-distillated water 65:25:4 (v/v/v) was used to separate polar lipids. The lipid spots were visualized with iodine, marked with a needle and identified by means of known standards. After iodine elimination, the marked areas were scraped and transferred to scintillation vials.

When phosphatidylcholines were to be fractionated further, the silica was transferred into a column plugged with glass wool and then eluted with 10 ml of chloroform-methanol (1:2) plus 10 ml of methanol. The distribution of the fatty acid incorporated into phosphatidylcholines was determined by digestion with phospholipase A_2 from *Crotalus adamanteus* (Koch Light Lab.) as described by VAN GOLDE and VAN DEENEN (15). Phosphatidyl-cholines were fractionated according to their degree of unsaturation by thin layer chromatography using 15 % silver nitrate/silica gel (w/w) plates and methanol-chloroform-water: 60:30:5 (v/v/v) as solvent, as described by KYRIAKIDES and BALINT (5). The spots were visualized under ultraviolet light by spraying the plate with 0.002 % dichlorofluorescein in methanol, marked and identified by means of known standards. The marked areas were scraped and transferred to scintillation vials. Phosphorous was determined by the method of BARTLETT (2). After elimination of the silver nitrate by thin layer chromatography using as solvent chloroform-methanol-water: 65:25:4 (v/v/v). Methyl esters were prepared (8) and analysed by gas liquid chromatography in a Hewlett Packard 5750 G gas chromatograph (6).

Radioactive material. Radioactive fatty acids were from Radiochemical Centre: 1-^{14}C palmitic acid, sp. actv. 58 mCi/mmol; 1-^{14}C stearic acid, sp. actv. 56 mCi/mmol; 1-^{14}C oleic acid, sp. actv. 59 mCi-mmol; 1-^{14}C linoleic acid, sp. actv. 58 mCi-mmol; and 1-^{14}C araquidonic acid, sp. actv. 58 mCi/mmol. To measure the radioactivity, 2 ml of scintillation liquid (composition: 150 mg of naftalen, 10 mg of PPO, 0.4 mg of POPOP, 1.4 ml of dioxane and 0.3 ml of water) were added to the vials containing the silicagel. Then, vials were capped, agitated and counted in a Nuclear Chicago scintillation spectrometer, model 6766. Counting efficiency was about 80 %.

Results

Incubation of liver homogenates from chick embryos with 10-100 nmoles of labeled palmitic acid for 1 hour at 37° C results in a 5 to 25 % incorporation of the label in phosphatidylcholines. The incorporation level remained constant for 6 hours of further incubation. The activity of the homogenates did not change after keeping them for 1-2 hours on ice. The incorporation of palmitic acid to phosphatidylcholines was 95 % inhibited by a final concentration of either 0.5 mM Ca^{++} or 2 mM Mg^{++}; EDTA at a final concentration of 0.1 mM did not affect the incorporation but it was 98 % inhibitory at 0.5 mM. The addition of ATP to the medium at a final concentration of 0.75 μM stimulated the incorporation 20 % but further increase on the ATP concentration produced inhibition of the incorporation (92 % inhibition at a final concentration of about 6 μM). No ATP or divalent cations were used thereafter.

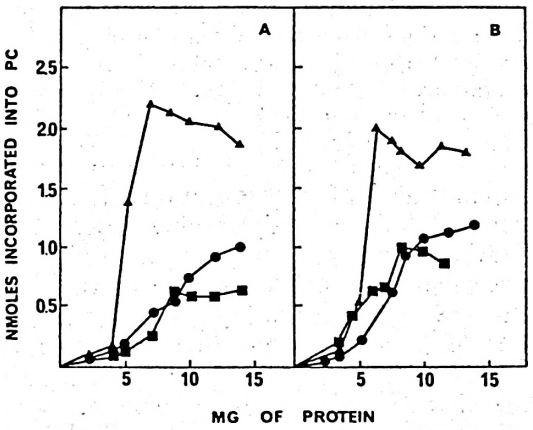

Fig. 1. *Effect of the protein concentration of homogenates of chick embryo liver on the incorporation of palmitic (A) and linoleic (B) acids into phosphatidylcholine.*
Mixtures containing 8.6 nmoles of sonicated labeled fatty acid and different amounts of protein, were incubated at 37° C for 1 hour in a final volume of 1 ml. Phosphatidylcholines were isolated as described in methods. Results are expressed as nmoles of fatty acid incorporated into phosphatidylcholine by liver homogenates from 13 (●), 17 (▲) and 21 (■) day-old chick embryos.

Figure 1 shows the variation of the incorporation rate of palmitic and linoleic acids into phosphatidylcholines with increasing concentrations of liver protein from 13, 17 and 21 day-old chick embryos. Increasing the amount of protein from 1 to 10 mg of protein/ml produced an increase in the incorporation of palmitic or linoleic acid to phosphatidylcholines; further increase in protein concentration, did not increase the incorporation to phosphatidylcholine. The maximum protein concentrations for which proportionally between protein concentration and incorporation were about 11, 8 and 9 mg/ml of protein for liver homogenates from 13, 17 and 21 day-old chick embryos, respectively. Eight mg of protein/ml were chosen to perform the rest of the experiments to give maximal incorporations. Figure 1 also shows that the profiles of variation of incorporation into phosphatidylcholine with protein concentration are very similar for palmitic and linoleic acids within each age studied (small variations were not reproducible in other experiments). The incorporation of palmitic or linoleic acids into phosphatidylcholine consistently showed an apparent 2-3 fold greater activity in liver homogenates from 17 day-old chick embryos than in those from 13 or 21 day-old ones.

The distribution of the incorporation of palmitic, stearic, oleic, linoleic and araquidonic acids among lipid classes did not vary with the time of incubation for each fatty acid in a given age of development. The patterns of distribution of the radioactivity incorporated among different lipid classes, were characteristic for each fatty acid but very similar throughout the development. Phosphatidylcho-

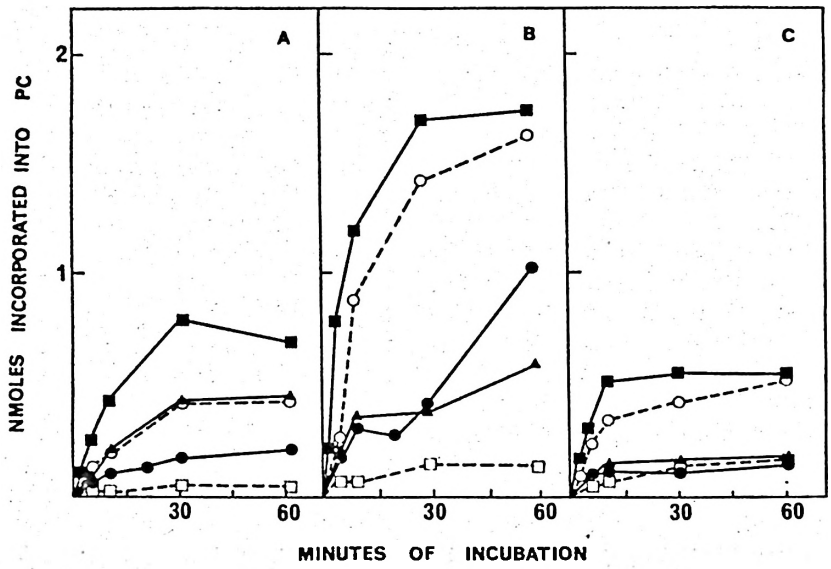

Fig. 2. *Time course of the incorporation of fatty acids into phosphatidylcholines by liver homogenates from 13 (A), 17 (B) and 21 (C) day-old chick embryos.*
Incubation mixtures containing 8.4 to 8.9 nmoles of labeled fatty acid and 8 mg of protein in a volume of 1 ml, were incubated at 37° C for the periods of time indicated. Phosphatidylcholines were isolated as described in methods. Results are expressed as nmoles of fatty acid incorporated into phosphatidylcholine. O····O, palmitic acid. □····□ stearic acid. ▲——▲, oleic acid. ■——■, linoleic acid. ●——●, araquidonic acid.

lines accumulate between 40 to 50 % of the total radioactivity incorporated into lipids after 1 hour of incubation with any of the fatty acids indicated above (data not shown).

Figure 2 shows the time courses of incorporation of palmitic, stearic oleic, linoleic and araquidonic acids into phosphatidylcholines. The incorporation increases during the first 10-30 min (depending on the fatty acid) then plateaus and remained constant. The rates of incorporation of palmitic and linoleic acids into phosphatidylcholines are always greater than the rates of incorporation of the other fatty acids, specially on liver homogenates from 17 day-old chick embryos. The rates of incorporation of oleic and araquidonic acids are very similar to each other and throughout the development.

The rates of incorporation of stearic acid into phosphatidylcholine are always very low (figure 2) when compared with the other fatty acids, in spite of the fact that stearoyl-phosphatidylcholines increase during development (3). Furthermore, formation of acyl-CoA at 2 min was shown to occur for every fatty acid studied; specifically, conversion of stearic acid into stearoyl-CoA took place to an extent of 39.1, 44.4 and 62.4 % for liver homogenates from 13, 17 and 21 day-old chick embryos, respectively (table I). Apparently the acyl-CoA formation is more active in liver homogenates from 21 day-old chick embryos but no significant variation between this percentage and the obtained for the other fatty acids within each age, could be demonstrated.

Fractionation of the phosphatidylcholine was made by thin layer chromatog-

Table I. *Acyl-CoA formation from lebeled fatty acids by liver homogenates from chick embryos.*
Three mg of liver protein were incubated with 30 nmoles of fatty acid in a final volume of 0.4 ml in Tris- KCL buffer (7). After 2 minutes of incubation at 37° C the reaction was stopped on ice and the free fatty acid was extracted 5 times with ether. Aqueous and ether extracts were concentrated and counted. Results are expressed as percentage of the recovered nmoles in the aqueous phase respect to the total nmoles recovered. The experiment was done in duplicate, the average and the ranges are represented in that figure.

Fatty acid	% of acyl-CoA		
	13 days	17 days	21 days
Palmitic acid (16:0)	41.2±1.7	51.9±3.8	69.4±0.4
Stearic acid (18:0)	39.1±4.1	44.4±5.5	62.4±1.2
Oleic acid (18:1)	44.7±0.2	38.3±3.1	55.0±6.7
Linoleic acid (18:2)	45.2±4.1	35.4±2.1	55.9±2.9
Araquidonic acid (20:4)	52.3±5.0	38.3±5.0	56.3±2.8

raphy impregnated with silver nitrate as described in methods. Three fractions were obtained in every case for liver homogenates from 13, 17 and 21 day-old chick embryos. Table II shows the fatty acid composition of the phosphatodylcholine fractionated: fraction 1 contains most of the oleic and linoleic acid (dienoic phosphatidylcholines), fraction 2 contains all the arachidonic acid and fraction 3 contains all the docosahexaenoic acid. All three fractions contained palmitic and stearic acids. No other fractions were detected. Corresponding results for 13 and 17 day-old chick embryos were in full agreement with the above ones; consequently, fractions 1, 2 and 3 will be referred as «dienoic», «tetraenoic» and «hexaenoic», respectively.

The distribution of labeled palmitic acid among the fractionated phosphatidylcholines as a function of the time of incubation did not change during the first hour of incubation. Approximately dienoic, tetraenoic and hexaenoic phosphatidylcholines incorporated about 50 %, 35 % and 10 %, respectively, of the total radioactivity incorporated to phosphatidylcholine. Figure 3 *A* shows the specific activity of the phosphatidylcholine fractions synthesized in the presence of labeled palmitic acid. Dienoic species showed nearly a 2 fold greater relative-rate of syn-

Table II. *Fatty acid composition of the phosphatidylcholines fractionated by thin layer chromatography impregnated with silver nitrate.*
Phosphatidylcholines were isolated from livers of 21 day-old chick embryos and fractionated ty thin layer chromatography over silica gel G impregnated with 15 % of silver nitrate as described in methods. Three fractions were obtained and after elimination of the silver nitrate by thin layer chromatography over silica gel, they were separately converted into methyl derivatives and analyzed by gas liquid chromatography. The fatty acids were identified by known standards. Composition is given in molar percentages. Similar qualitative results were obtained by fractionation of liver phosphatidylcholines from 13 or 17 day-old chick embryos.

Fatty acid	Molar percentages			
	Total	Fr.1 (Rf.0.54)	Fr.2 (Rf.0.36)	Fr.3 (Rf.0.2)
Palmitic acid (16:0)	25	24	21	47
Stearic acid (18:0)	27	27	33	12
Oleic acid (18:1)	13	18	—	—
Linoleic acid (18:2)	23	32	—	2
Araquinonic acid (20:4)	10	—	46	2
Docosahexaenoic acid (22:6)	7	—	—	37

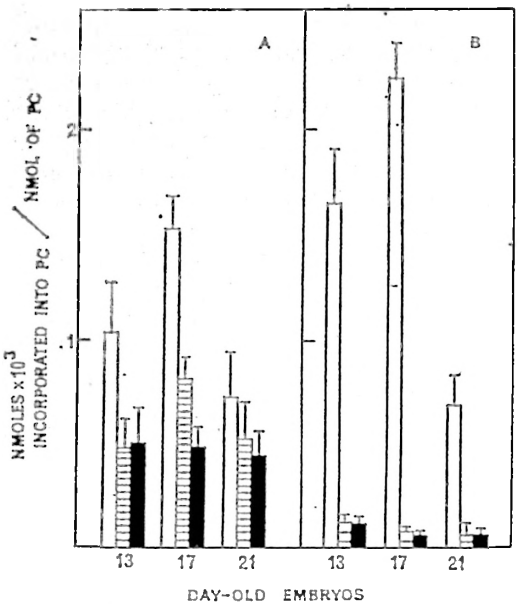

Fig. 3. *Incorporation of palmitic (A) and linoleic (B) acids into fractionated phosphatidylcholines.*
Mixtures containing 34 nmoles of labeled fatty acid and 8 mg of protein in a total volume of 1 ml, were incubated at 37° C for 1 hour. Phosphatidylcholines were isolated and fractionated as described in methods. Radioactivity incorporated and phosphorus were determined as described. Specific activities are expressed as nmoles of fatty acid incorporated/nmol of phosphatidylcholine. The average value and the range from 3 independent experiments is represented in the figure. Phosphatidylcholines containing 0.1 or 2 double bonds are represented as white bars; phosphatidylcholines containing 4 double bonds are represented as hatched bars and phosphatidylcholines containing 6 double bonds are represented as black bars. Liver homogenates were obtained from 13, 17 and 21 day-old chick livers.

thesis than tetraenoic species in liver from 13 and 17 day-old chic embryos. The specific activity of the palmitic acid labeled dienoic and tetraenoic phosphatidylcholines increases from 13 to 17 days and decreases from 17 to 21 days. The specific activity of the hexaenoic phosphatidylcholines remained constant during development. Figure 3 *B* shows the specific activity of the phosphatidylcholine fractions synthesized in the presence of labeled linoleic acid. Dienoic phosphatidylcholines were the only fraction which appeared labeled. Phosphatidylcholine from 17 day-old chick embryos showed the highest specific activity.

Table III. *Palmitic and linoleic acid incorporated into the position 2 of the phosphatidylcholine molecule.*
Mixtures containing 17 nmoles of sonicated labeled fatty acid and 8 mg liver protein in a final volume of 1 ml were incubated at 37° C for 10, 60 and 120 minutes. Phosphatidylcholine isolation and digestion were as described in methods. The data was calculated according to the formula: cpm in free fatty acids/cpm in free fatty acids + cpm in lysophosphatidylcholine × 100. The average and ranges of the three determinations are given.

Chick-embryos age	% of radioactivity released in free fatty acids	
	Palmitic acid	Linoleic acid
13 day-old	24 ± 3	86 ± 2
17 day-old	15 ± 1	86 ± 2
21 day-old	21 ± 3	94 ± 1

To determine the distribution of the incorporated palmitic and linoleic acids into the molecule of phosphatidylcholine, the fatty acid in the position 2 was hydrolyzed with phospholipase A and the radioactivity released as free fatty acid and lysophosphatidylcholine was measured as described in methods. Table III shows the percentage of the total radioactivity released recuperated in the free fatty acid. Fifteen to 25 % of the palmitic acid incorporated to phosphatidylcholines and 85 to 95 % of the linoleic acid incorporated to phosphatydylcholines were released by phospholipase A digestion. No significant variations in the distribution of the palmitic or linoleic acids incorporated were found during development.

Discussion

This study demonstrates that liver homogenates from 13, 17 and 21 day-old chick embryos were able to use palmitic, stearic, oleic, linoleic and arachidonic acids to synthesize phosphatidylcholine.

Palmitic and linoleic acids were incorporated into phosphatidylcholines to a higher extent than the other fatty acids studied (fig. 2). Fractionation of phosphatidylcholines synthesized *in vitro* according to their unsaturation degree (table II and fig. 3) and positional analysis of incorporated palmitic and linoleic acids (table III) make it apparent that both dienoic and tetraenoic molecular species are synthesized with palmitoyl chains predominantly located at position sn-1, whereas linoleoyl residues are linked almost exclusively to position sn-2. These results suggest a preferential synthesis of 1-palmitoyl-2-linoleoyl-sn-glycerophosphorylcholine and, to a lesser extent, of 1-palmitoyl-2-araquidonoyl-sn-glycero-3-phosphorylcholine molecules. Apparently, liver homogenates from 17 day-old chick embryos were 2-3 fold more active than the others in incorporating palmitic and linoleic acids into those molecular species (figs. 1, 2 and 3). These results correlate with the increase in dienoic species that takes place during embryonic development (1) and could be explained in terms of an increased synthesis of dienoic molecular species around the 17th day of development. Either the KENNEDY pathway (4, 12, 16) or deacylation-reacylation reactions (13, 14) could explain the palmitic and linoleic acids incorporation into phosphatidylcholine.

The amount of stearic acid in phosphatidylcholine increases during development (1, 9, 10) and yet the incorporation of stearic acid into phosphatidylcholine is very low when compared to other fatty acids (fig. 2). Since stearoyl-coA is formed at the same rate that any of the other fatty acids (table I), no explanation can be offered at present to account for such low incorporation.

Resumen

Los ácidos marcados palmítico, esteárico, oleico linoleico y araquidónico se incorporaron a fosfatidilcolinas durante la incubación con homogenados de hígados de embriones de pollo de 13, 17 y 21 días. El ácido palmítico se incorporaba preferentemente a las fosfatidilcolinas dienoicas y tetraenoicas. Los ácidos palmítico y linoleico marcados se encontraron específicamente en las posiciones 1 y 2 de la molécula de fosfatidilcolina, respectivamente. En nuestras condiciones *in vitro*, la incorporación de ácidos palmítico o linoleico fue 2-3 veces más alta en homogenados de hígado de embriones de pollo de 17 días que en homogenados de hígados de embriones de pollo de de 13 o de 21 días.

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. COLEMAN, R. and BELL, R. M.: *J. Biol. Chem.*, 252, 3050-3056, 1977.
5. KYRIAKIDES, E. C. and BALINT, J. A.: *J. Lipid. Res.*, 9, 142-143, 1968.
6. MADARIAGA, M. A., MUNICIO, A. M. and RIBERA, A.: *Comp. Biochem. Physicl.*, 36, 271-278, 1970.
7. MARCEL, Y. L. and SUZUE, G.: *J. Biol. Chem.*, 247, 4433-4436, 1972.
8. METCALFE, L. E. and SCHMITZ, A. A.: *Anal. Chem.*, 33, 363-364, 1961.
9. MIYAMOTI, K., STEPHANIDES, L. M. and BERHSON, J.: *J. Lipid Res.*, 7, 664-670, 1966.
10. NOBLE, R. C. and MOORE, J. H.: *Can. J. Biochem.*, 45, 627-639, 1967.

11. LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. and RANDALL, R. J.: *J. Biol. Chem.*, **193**, 265-275, 1951.

12. VANCE, D. E. and DAHLKE, R. M.: *Can. J. Biochem.*, **53**, 950-957, 1975.

13. VAN DEN BOSCH, H., SLOTBOOM, A. J. and VAN DEENEN, L. L. M.: *Biochim. Biophys. Acta*, **144**, 613-623, 1969.

14. VAN DEN BOSCH, H., SLOTBOOM, A. J. and VAN DEENEN, L. L. M.: *Biochim Biophys.*, **176**, 623-634, 1969.

15. VAN GOLDE, L. M. G. and VAN DEENEN, L. L. M.: *Chem. Phys. Lipids.*, **1**, 157-164, 1967.

16. WEISS, S. B., SMITH, S. W. and KENNEDY, E. P.: *J. Biol. Chem.*, **231**, 53-64, 1958.