Metabolism of Phosphatidylcholine in Brain and Liver of Developing Rats

NOBUKAZU CHIDA and TSUNEO ARAKAWA

Department of Pediatrics, Tohoku University School of Medicine, Sendai

CHIDA, N. and ARAKAWA, Ts. *Metabolism of phosphatidylcholine in brain and liver of developing rats.* Tohoku J. exp. Med., 1971, 104 (4), 359-371 — Incorporation of CDP-choline-1, 2-¹⁴C into phosphatidylcholine by brain homogenate and microsome was found to be higher than that of liver before and during active myelination period in rats. Incorporation of S-adenosylmethionine-methyl-¹⁴C into phosphatidylcholine by brain microsome was about 1/20 of that by liver microsome in two-week-old rats. These results indicate that in developing rat brain, phosphatidylcholine may be synthesized by transmethylation, to some extent, in addition to the major pathway *via* CDP-choline. ——— phosphatidylcholine synthesis; brain; myelination

Phosphatidylcholine is widely distributed in both gray and white matter of the brain (McIlwain 1966). It is also known that phosphatidylcholine is one of the major components of membrane structure including myelin sheath (O'Brien 1965, Arakawa *et al.* 1969). At birth, brain is more than twice as large as liver in rat as well as in human (Nesbitt 1966), and undergoes rapid growth, associated with active lipid synthesis and myelin formation (Smith 1967, Cuzner and Davison 1968). It is suggested that a number of enzymes concerning lipid metabolism show an increase in their activity before and during active myelination period (McMurray 1964).

The purpose of this experiment was to study the *in vivo* and *in vitro* biosynthesis of phosphatidylcholine in developing rat brain with special reference to two major pathways:

- (1) CDP-choline + D- α , β -diglyceride $\longrightarrow$ phosphatidylcholine (Kennedy and Weiss 1956)
↑
(CDP-choline: 1, 2-diglyceride choline phosphotransferase)
- (2) phosphatidylethanolamine + S-adenosyl-L-methionine
 $\longrightarrow$ phosphatidylcholine (Bremer and Greenberg 1961, Bjørnstad and Bremer 1966, Bremer *et al.* 1960, Bremer and Greenberg 1960)
↑
(Phosphatidylethanolamine: adenosylmethionine methyltransferase)

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MATERIALS AND METHODS

The Wistar strain rats were used in this experiment and fed on a standard diet purchased from the Oriental Co., Tokyo, Japan. L-methionine-methyl- ^{14}C (specific activity 10.3 mCi/mmole) and choline-methyl- ^{14}C (specific activity 5.2 mCi/mmole) were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. Choline-methyl- ^3H (specific activity 6,700 mCi/mmole) and methionine-methyl- ^{14}C (specific activity 56.8 mCi/mmole) were obtained from Nuclear Chicago, U.S.A. S-adenosyl-L-methionine-methyl- ^{14}C (specific activity 49 mCi/mmole) was purchased from the Radiochemical Centre, Amersham, England. CDP-choline-1,2- ^{14}C (specific activity 2.2 mCi/mmole) was a gift from Takeda Chemical Co., Osaka, Japan.

In vivo studies: all radioactive materials were diluted with 0.9% saline and administered intraperitoneally (Bjørnstad & Bremer, 1966).

For a single tracer study, methionine-methyl- ^{14}C or choline-methyl- ^{14}C was given intraperitoneally at a dose of 5 μCi per 20 g body weight. For a double tracer study, a mixture of methionine-methyl- ^{14}C and choline-methyl- ^3H (10:1 by radioactivity) was administered. Animals were killed by exsanguination, and organs were homogenized in a small volume of ice-cold water. Three volumes of 10% trichloroacetic acid (TCA) were added to the homogenate and mixed well and centrifuged. The precipitates were rinsed twice with three volumes of 5% TCA and centrifuged. Supernatants were combined and stored as "TCA soluble fraction".

Precipitates were extracted with a mixture of chloroform and methanol (2:1, v/v), and the lipid fraction was evaporated to dryness. Fractionation and purification of lipids were carried out by DEAE-cellulose column chromatography and thin layer chromatography (TLC), according to Rouser *et al.* (1965).

Spots on TLC plates were identified with standards (sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, cerebroside, and sulfatide which were purchased from Applied Science Lab., U.S.A.), and scraped off into counting vials with 4% Cab-O-sil toluene scintillator according to Snyder and Stephens (1962) to measure radioactivity by means of Aloka Liquid Scintillation Counter, Type LSC-601, Japan Radiation & Medical Electronics, Inc. Tokyo, Japan. TCA soluble fractions were dried up under heating lamp, cooled and dissolved in 0.2 ml of methanol and radioactivity was determined in Bray's solution (Bray 1960) using Aloka Liquid Scintillation Counter. For simultaneous determination of ^3H and ^{14}C activity in experiments in which both methionine-methyl- ^{14}C and choline-methyl- ^3H were used, samples were treated in the same way as described above and radioactivity of ^3H and ^{14}C was determined by screenout method in Aloka Liquid Scintillation Counter.

In vitro studies: The microsome fractions were prepared according to Brady *et al.* (1965). The incubations were carried out in test tubes at 37°C. Each tube contained 50 μmoles of Tris buffer (PH 8.0), 10 μmoles of MgCl_2 , 5 μmoles of reduced glutathione, 0.5 μCi of CDP-choline-1,2- ^{14}C or S-adenosyl-L-methionine-methyl- ^{14}C and 0.3 ml of microsome fraction (about 20 mg as protein) in 0.2 M Tris buffer or 0.5 ml of homogenate in 0.2 M Tris buffer (PH 8.0) which was prepared by centrifugation at $1,000 \times g$ for 10 min. When methionine-methyl- ^{14}C was used instead of S-adenosyl-methionine-methyl- ^{14}C , 10 μmoles of ATP was also added to the incubation mixture described above, according to Gibson *et al.* (1961). At the end of incubation, the reaction was stopped by addition of 10 volumes of a mixture of chloroform and methanol (2:1, v/v) and mixed well. Chloroform fraction was washed three times with 0.9% saline. Fractionation of phospholipids was done as described above. When S-adenosyl-L-methionine-methyl- ^{14}C was used, phosphatidylcholine was further hydrolyzed and choline was precipitated as the Reineckate salt. Decomposition of choline Reineckate and determination of radioactivity in choline moiety was carried out according to Bremer and Greenberg (1961).

RESULTS

In vivo incorporation of methionine-methyl- ^{14}C into brain and liver phosphatidylcholine: In 2-week-old rats a significant incorporation of methionine-methyl- ^{14}C into liver phosphatidylcholine was observed as early as 30 minutes after the intraperitoneal injection, this result being in agreement with others' results (Bjørnstad

TABLE 1. Transport of methionine-methyl- ^{14}C into brain and incorporation of methionine-methyl- ^{14}C into phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin of the brain and liver in 2-week-old rats. The radioactive material was injected intraperitoneally

	Time after injection	TCA soluble fraction cpm/100 mg wet weight	Phosphatidylcholine		Phosphatidylethanolamine cpm/100 mg wet weight	Sphingomyelin cpm/100 mg wet weight
			cpm/100 mg wet weight	cpm/100 γ . phosphorus		
Brain	30 minutes	2,141	73	146	3	2
	60 "	2,942	143	376	6	32
	2 hours	2,358			5	76
	4 "	2,291	271	301	4	76
	8 "	2,139	693	778	20	71
	24 "	2,733	1,881	3,762	44	106
Liver	30 minutes	10,174	27,609	62,700	592	3,948
	60 "	11,992	23,098	56,336	258	3,738
	2 hours	7,946	24,648	60,237	218	4,226
	4 "	8,694	24,724	50,457	102	2,948
	8 "	9,480	18,080	53,176	112	2,806
	24 "	7,590	4,356	15,725	56	983

Fig. 1 Incorporation of methionine-methyl- ^{14}C into liver and brain phosphatidylcholine in 2-week-old rats. The radioactive material was injected intraperitoneally.

○—○ Brain phosphatidylcholine △—△ Liver phosphatidylcholine

and Bremer 1966) (Table 1, Fig. 1). The radioactivity of liver sphingomyelin was about 1/7 of phosphatidylcholine. The radioactivity of TCA soluble fractions of brain, which are thought to represent roughly free methionine-methyl- ^{14}C , showed about 1/5 of those of livers, remaining constant, probably due to the regulation by blood brain barrier (Neame 1968).

Incorporation of methionine-methyl- ^{14}C into brain phosphatidylcholine was very low soon after the injection, but increased steadily to about 1/2 of liver phosphatidylcholine one day after the injection.

In vivo incorporation of choline-methyl- ^{14}C into brain phosphatidylcholine: The radioactive material was injected intraperitoneally into 2-week-old rats and time courses for the choline-methyl- ^{14}C incorporation into brain and liver phosphatidylcholine are shown in Table 2 and Fig. 2. Maximum specific activity of liver

TABLE 2. *Transport of choline-methyl- ^{14}C into brain and incorporation week-old rats (the radioactive material*

Time after injection	Serum		TCA soluble fraction cpm/whole brain
	TCA soluble fraction cpm/ml	Phosphatidylcholine cpm/ml	
15 minutes	91,580	93	2,510
30 "	77,144	252	2,820
60 "	72,244	626	2,793
2 hours	78,444	9,288	2,631
4 "	51,026	7,686	2,570
8 "	62,200	17,834	3,666
24 "	40,200	3,834	2,692
2 days	9,956	3,448	2,412
3 "	10,250	4,950	2,377

Fig. 2. Incorporation of choline-methyl- ^{14}C into liver and brain phosphatidylcholine in 2-week-old rats. The radioactive material was injected intraperitoneally.

○—○ Brain phosphatidylcholine △—△ Liver phosphatidylcholine

phosphatidylcholine was found about 4 hours after the injection. A rapid equilibration of liver and plasma phosphatidylcholine has been reported to take place (Björnstad and Bremer 1966). In our present experiment maximum radioactivity of serum phosphatidylcholine was found about one hour after that of liver phosphatidylcholine. Radioactivity of the TCA soluble fraction of brain, which was thought to be due mostly to free choline-methyl- ^{14}C , was far less than that of serum, but in similarity with the case with methionine-methyl- ^{14}C , the level of the radioactivity in brain remained almost constant, indicating the possible regulation by blood brain barrier.

Radioactivity of brain phosphatidylcholine increased steadily after the choline-methyl- ^{14}C administration in terms of both specific activity and radioactivity per tissue wet weight. Liver phosphatidylcholine, on the other hand, decreased rapidly in radioactivity 8 hours after the injection, getting close to that of brain

of choline-methyl- ^{14}C into brain- and liver-phosphatidylcholine of two-weeks injected intraperitoneally)

Brain		Liver	
Phosphatidylcholine		Phosphatidylcholine	
cpm/100 mg wet weight	cpm/100 γ phosphorus	cpm/100 mg wet weight	cpm/100 γ phosphorus
198	95	5,951	13,391
217	129	10,518	8,347
315	216	8,483	22,577
391	272	6,153	26,000
727	478	20,350	37,779
1,589	986	15,980	29,134
3,245	1,602	4,771	8,849
3,120	1,928	1,793	3,804
4,473	2,188	1,990	4,265

TABLE 3. *Incorporation of CDP-choline-1, 2- ^{14}C into phosphatidylcholine by microsome from brain and liver of 2-week-old rats*

	cpm/10 mg protein
Intact brain microsome fraction	112,372
Boiled brain microsome fraction	136
Intact liver microsome fraction	91,780
Boiled liver microsome fraction	2,458

phosphatidylcholine, as is shown in Table 2.

Incorporation of CDP-choline-1,2- ^{14}C into phosphatidylcholine: Brain microsome fractions or homogenates were found to have as high activity as in liver microsome fractions or homogenates. Fetus (19 days gestation) and one to two week-old rats showed higher activities in brain than in liver. The activity increased after birth, showing a peak at two weeks after birth and declining slowly thereafter as

TABLE 4. *Incorporation of CDP-choline-1, 2-¹⁴C into phosphatidylcholine by brain and liver microsome fractions of rats with various age groups*

Age of animal	Brain cpm/10 mg protein	Liver cpm/10 mg protein
Fetus (19 days gestation)	85,908	74,668
"	79,162	67,319
2 weeks of life	112,372	91,780
"	131,083	80,313
"	222,014	
3 weeks of life	74,692	
"	65,493	
"	83,571	
Adult	56,184	
"	40,388	
"	53,943	
"	54,404	

TABLE 5. *Incorporation of CDP-choline-1, 2-¹⁴C into phosphatidylcholine by brain- and liver-homogenates of rats with various age groups*

Age of animal	Brain cpm/10 mg protein	Liver cpm/10 mg protein
4 days	64,144	29,370
2 weeks	26,482	20,857
"	28,418	17,817
4 weeks	16,474	
"	21,914	19,277
Adult	6,827	12,425
"	12,157	18,152
"		18,548
"		9,591

shown in Tables 3, 4 and 5. McMurray (1964) reported that CDP-choline was actively incorporated into alkali-labile phospholipids by brain homogenate of developing rats, although they did not purify phosphatidylcholine. Our present data are in fairly good agreement with theirs.

Incorporation of S-adenosylmethionine-methyl-¹⁴C or methionine-methyl-¹⁴C into phosphatidylcholine by brain microsome or homogenate: Time course of the phosphatidylcholine formation from S-adenosylmethionine by rat brain microsome is shown in Fig. 3. In Table 6 was shown the incorporation of S-adenosylmethionine-methyl-¹⁴C into phosphatidylcholine and choline moiety by intact and boiled microsome fraction of brain and liver of two-week-old rats. Brain microsome fraction showed about 1/20 of activity of liver microsome. S-adenosylmethionine incorporation was investigated using fetus (19 days gestation), one-, two- and three-week-old rats and adult rats (Table 7). Relatively high incorporation rates were seen in one- and two-week-old rats as compared to fetus and adults.

Similar results were obtained in the methionine incorporation into phosphatidylcholine by brain- and liver-homogenates as shown in Table 8.

Fig. 3. Time course for incorporation of S-adenosylmethionine-methyl- ^{14}C into phosphatidylcholine by brain microsome fraction of 2-week-old rats.

TABLE 6. Incorporation of S-adenosylmethionine-methyl- ^{14}C into phosphatidylcholine and choline by microsome fractions of liver and brain of 2-week-old rats

	Phosphatidylcholine cpm/15 mg protein	Choline cpm/15 mg protein
Intact brain microsome fraction	862	690
Boiled brain microsome fraction	378	290
Intact liver microsome fraction	11,278	9,764
Boiled liver microsome fraction	230	173

TABLE 7. Incorporation of S-adenosylmethionine-methyl- ^{14}C into phosphatidylcholine by brain- and liver-microsome fractions in rats with various age groups

Age of animal	Brain cpm/10 mg protein	Liver cpm/10 mg protein
Fetus (19 days gestation)	147	
"	163	
"	216	
1 week of life	924	17,276
"	882	
"	695	
2 weeks of life	574	14,699
"	733	
"	501	18,621
Adult	155	
"	170	
"	122	

In vivo incorporation of methionine-methyl- ^{14}C into whole brain phosphatidylcholine: Incorporation of methionine-methyl- ^{14}C into whole brain phosphatidylcholine was compared with that of whole liver phosphatidylcholine at various stages of development (Fig. 4.)

$$N = \frac{\text{Radioactivity of whole brain phosphatidylcholine}}{\text{Radioactivity of whole liver phosphatidylcholine}} \times 100$$

The N values at 2 hours after the methionine-methyl- ^{14}C injection were significantly

TABLE 8. *Incorporation of methionine-methyl-¹⁴C into phosphatidylcholine by brain- and liver-homogenates in rats with various age groups*

Age of animal	Brain cpm/10 mg protein	Liver cpm/10 mg protein
2 weeks of life	429	2, 218
"	171	2, 251
"	289	4, 318
"	181	3, 515
4 weeks of life	116	
"	88	
"	97	
Adult	75	
"	76	
"	133	
"	233	
"	141	
"	188

Fig. 4. Comparison between incorporation of methionine-methyl-¹⁴C into whole brain phosphatidylcholine and that into whole liver phosphatidylcholine in rats with various age groups. The radioactive material was injected intraperitoneally.

$$N = \frac{\text{Radioactivity of whole brain phosphatidylcholine}}{\text{Radioactivity of whole liver phosphatidylcholine}} \times 100$$

●—● N at 2 hours after administration ○—○ N at 24 hours after administration

higher in fetus and suckling rats of one- to three-week-old as compared to mature ones, indicating that much larger amounts of methionine were utilized for phosphatidylcholine synthesis in suckling rats than in adults.

In vivo incorporation of simultaneously injected methionine-methyl-¹⁴C and choline-methyl-³H into brain- and liver-phosphatidylcholine: Both the radioactive materials were injected intraperitoneally into rats of 21 days gestation. Time curves of ³H/¹⁴C of phosphatidylcholine for pregnant mother and fetus are shown in Fig. 5. ³H/¹⁴C of brain phosphatidylcholine of mother was remarkably high, indicating that major pathway of phosphatidylcholine synthesis in mother brain

Fig. 5. Choline-methyl- ^3H and methionine-methyl- ^{14}C were injected simultaneously into a rat of 21 days gestation. The ratio of ^3H to ^{14}C of phosphatidylcholine was examined on serum of the pregnant rat and on brain, and liver of the dam and fetus.

$$^3\text{H}/^{14}\text{C} = \frac{^3\text{H of phosphatidylcholine}}{^{14}\text{C of phosphatidylcholine}}$$

△—△ Fetal liver

●—● Maternal brain

○—○ Fetal brain

○—○ Maternal serum

▲—▲ Maternal liver

is *via* CDP-choline pathway. In fetus, on the other hand, $^3\text{H}/^{14}\text{C}$ was relatively low as compared to their mothers.

Relatively low $^3\text{H}/^{14}\text{C}$ was found in one- or two-week-old rats (Fig. 6).

DISCUSSION

It has been known that entry of amino acids into brain is under control of blood brain barrier (Neame 1968). Our data on the transport of methionine into brain are in good agreement with the concept. The present experiment also showed that the transport of choline into brain was, similarly to methionine, remarkably restricted, indicating that choline transport was also under a sort of control of blood brain barrier in developing rat brain as well as in mature ones.

Fig. 6. Choline-methyl- ^3H and methionine-methyl- ^{14}C were injected simultaneously into rats with various age groups, including a rat of 21-day gestation. The radioactivity of phosphatidylcholine was estimated 2 hours after the injection.

$$^3\text{H}/^{14}\text{C} = \frac{^3\text{H of phosphatidylcholine}}{^{14}\text{C of phosphatidylcholine}}$$

Methionine was poorly incorporated into brain phosphatidylcholine, the result being similar to the results of extrahepatic tissues by other investigators (Bjørnstad and Bremer 1966). However, relatively large amounts of methionine were incorporated into brain phosphatidylcholine before and during the active myelination period. There are two possible explanations for this phenomenon: (a) In young rats, entry of amino acids into brain are thought to be less restricted than in mature ones because of incomplete blood brain barrier. Thus more methionine will enter brain to be utilized for phosphatidylcholine synthesis as discussed below. (b) Intact phosphatidylcholine which was synthesized by transmethylation in liver might have entered brain through incomplete blood brain barrier in young rats.

Two pathways are known for the *de novo* synthesis of phosphatidylcholine: A) Conversion of phosphatidylethanolamine to phosphatidylcholine by a step-wise methylation (Bremer 1961). B) Incorporation of choline into phosphatidylcholine *via* phosphorylcholine and CDP-choline (Kennedy and Weiss 1956).

Pathway A is known to be very active in liver but far less active in extrahepatic tissue (Bremer and Greenberg 1961, Bjørnstad and Bremer 1966, Marshall *et al.* 1965). In our experiment, pathway B was very active in brain

of growing rats. The activity increased after birth with a peak at about two weeks after birth and declined slightly thereafter, in agreement with McMurray (1964). It is noteworthy that, in developing rats, both homogenate and microsome of brain showed higher activity *in pathway B* than those of liver. In spite of the high incorporation of CDP-choline into phosphatidylcholine *in vitro*, *in vivo* incorporation of choline into brain phosphatidylcholine was found to be far less than that in liver probably because transport of choline into brain is restricted, similarly to methionine. On the other hand, an active *in vivo* incorporation of choline into brain phosphatidylcholine was reported by Ansell and Spanner (1968). However, in their experiment, choline was administered directly intracranially by-passing blood brain barrier, whereas in our experiment, radioactive choline was given intraperitoneally.

The present experiment also showed that the *in vitro* methionine incorporation into phosphatidylcholine by brain homogenate or microsome was significantly *low in adult rats* and this finding was in agreement with other investigators' results (Bremer and Greenberg 1961, Marshall *et al.* 1965). In contrast to mature ones, *in vitro* methionine incorporation was *relatively high in young rats*, suggesting that phosphatidylcholine may be synthesized *via* Bremers' pathway (Bremer and Greenberg 1961) to some extent, in addition to Kennedy's (1956) at least before and during the active myelination period. Results obtained by the double tracer study using choline-methyl- ^3H and methionine-methyl- ^{14}C *in vivo* in our present experiment seem to support this possibility.

CONCLUSION

Phosphatidylcholine biosynthesis in developing rat brain was studied *in vivo* and *in vitro*. Transport of choline into brain was, similarly to that of methionine, found to be remarkably restricted, probably due to the regulation by blood brain barrier. Radioactivity of brain phosphatidylcholine after the intraperitoneal injection of methionine-methyl- ^{14}C was very low for the first several hours and then continued to increase slowly, while that of liver phosphatidylcholine was remarkably high soon after injection, declining thereafter. The mode of *in vivo* incorporation of choline into brain phosphatidylcholine was very similar to that of methionine; it was very low soon after administration, but increased steadily, getting close to that of liver phosphatidylcholine one day after.

In vitro incorporation of S-adenosylmethionine-methyl- ^{14}C into phosphatidylcholine by brain microsome was about 1/20 of that by liver microsome in two-week-old rats. Relatively high rate of methionine incorporation, though far less than that of liver, was observed in one- and two-week-old rats as compared to mature ones.

Incorporation of CDP-choline-1,2- ^{14}C into phosphatidylcholine by brain homogenate or microsome fraction was remarkably high; the activity was found to be higher than that with liver before and during the active myelination period.

Simultaneous administration of choline-methyl- ^3H and methionine-methyl- ^{14}C was performed in different age groups with the aim to evaluate the two pathways of phosphatidylcholine biosynthesis: (1) CDP-choline + D- α , β -diglyceride $\longrightarrow$ phosphatidylcholine and (2) phosphatidylethanolamine + S-adenosyl-L-methionine $\longrightarrow$ phosphatidylcholine. $^3\text{H}/^{14}\text{C}$ of liver phosphatidylcholine was almost constant, showing no significant differences among different age groups. In brain, however, $^3\text{H}/^{14}\text{C}$ was low in fetus and suckling rats as compared to that in mature ones. These results indicate that, in developing rat brain, phosphatidylcholine may be synthesized by transmethylation to some extent, in addition to the major pathway via CDP-choline.

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