Role of a Specific Choline Pool in Sphingomyelin Synthesis in Rat Heart

By ZOLTÁN KISS

*Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences,
6701 Szeged, P.O. Box 521, Hungary*

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Sphingomyelin synthesis was studied in slices of rat heart by using [*Me*-¹⁴C]choline, [1,2-¹⁴C]ethanolamine, *S*-adenosyl-L-[¹⁴C]methionine and [³²P]P_i as precursors. In the presence of both [*Me*-¹⁴C]choline and [³²P]P_i the ratio of the specific radioactivities of ¹⁴C and ³²P in phosphatidylcholine was greater than in sphingomyelin at all the times studied. This suggested that synthesis of phosphatidylcholine and sphingomyelin *de novo* did not involve the utilization of a common pool of cytidine diphosphate choline. In addition, studies with [1,2-¹⁴C]ethanolamine and *S*-adenosyl-L-[¹⁴C]methionine indicated that a quantitatively significant pool of choline, derived from these precursors, was selectively utilized for sphingomyelin formation. This pool was not represented by phosphatidylcholine formed by methylation of phosphatidylethanolamine or by other pathways.

The exclusive role of cytidine diphosphate choline as precursor in the biosynthesis of sphingomyelin (Kennedy & Weiss, 1956; Sribney & Kennedy, 1958; Brady *et al.*, 1965; Sribney, 1968; Fujino *et al.*, 1968) is the subject of debate. In mouse cells transformed with SV 40 virus, a new pathway of sphingomyelin synthesis via phosphatidylcholine has been demonstrated that involves the transfer of the phosphorylcholine group (Marggraf *et al.*, 1972; Diringer *et al.*, 1972; Diringer, 1973; Diringer & Koch, 1973; Marggraf & Anderer, 1974). It has also been shown that phospholipase C (EC 3.1.4.3) from *Clostridium perfringens* may catalyse this transfer (Kanfer & Spielvogel, 1975). A similar mechanism could occur in mammalian tissues, since they are known to contain phospholipase C (Williams *et al.*, 1973). In the liver, a considerable amount of choline that is incorporated into sphingomyelin is synthesized endogenously and is not derived from the pool of free choline (Spitzer *et al.*, 1969).

The present work shows that the synthesis of sphingomyelin in rat heart also involves an endogenously synthesized pool of choline that is selectively incorporated into sphingomyelin. The possible nature of this choline pool is discussed.

Experimental

Materials

Chemicals. [*Me*-¹⁴C]Choline (sp. radioactivity, 43.2 Ci/mol) and [1,2-¹⁴C]ethanolamine (sp. radioactivity, 2 Ci/mol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. *S*-Adenosyl-L-

[¹⁴C]methionine (sp. radioactivity, 56.7 Ci/mol) was obtained from New England Nuclear Corp., Boston, MA, U.S.A., and carrier-free [³²P]P_i from the Isotope Institute of the Hungarian Academy of Sciences, Budapest, Hungary. All the other chemicals were of analytical grade.

Animals. Male Wistar rats (200–250 g) were used that had been starved overnight.

Methods

Incubation conditions. Heart slices with an average thickness of 0.25 mm were shaken (120–130 mg wet wt./ml) in bicarbonate-buffered medium (122 mM-NaCl, 3 mM-KCl, 1.2 mM-MgSO₄, 1.3 mM-CaCl₂, 5 mM-glucose and 25 mM-NaHCO₃) that was supplemented with 50 mM-Tris/HCl, pH 7.4. The other additions were [³²P]P_i (150 μCi/ml) and either [*Me*-¹⁴C]choline (1 μCi/ml) or [1,2-¹⁴C]ethanolamine (1 μCi/ml).

Incubations were at 37°C, and at appropriate time intervals samples were withdrawn and homogenized in 10 ml of chloroform/methanol (2:1, v/v). In pulse-chase experiments, slices were thoroughly washed with ice-cold bicarbonate buffer after incubation for 80 min with [³²P]P_i and then reincubated in isotope-free bicarbonate buffer at 37°C.

For studies on the incorporation of labelled *S*-adenosyl-L-methionine, the standard incubation mixture contained 17.6 nM-*S*-adenosyl-L-[¹⁴C]methionine, 50 mM-Tris/HCl buffer, pH 7.4, and homogenates of heart, liver, kidney, lung and brain cortex (2.1–2.9 mg of protein) in a final volume of 0.5 ml. Mixtures were incubated in a shaking water bath for

20 min at 37°C. Reactions were stopped by adding 10 ml of chloroform/methanol (2:1, v/v).

Chromatography. The extraction of lipids and their separation by t.l.c. was performed as described by Kiss (1976). Parallel plates were run to determine the radioactivity and the phosphorus content of phospholipids. The areas containing lipid were detected by brief exposure to I_2 vapour and were either scraped into counting vials or charred with 50% (v/v) H_2SO_4 at 180°C for 60 min. The hydrolysis of isolated sphingomyelin and phosphatidylcholine was carried out in 6M-HCl by the method of Robins *et al.* (1956). After filtration, portions of the hydrolysates were spotted on Whatman 3MM paper and the papers were run in acetone/water/12M-HCl (100:20:1, by vol.). The R_F values for choline and ethanolamine were 0.28–0.31 and 0.78–0.83 respectively.

Measurement of radioactivity. ^{32}P and ^{14}C radioactivity of double-labelled phospholipids was determined in toluene scintillation fluid [4% (w/v) 2,5-diphenyloxazole and 0.2% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene] with the use of the appropriate channels. The radioactivity measurements were repeated four times after each period of 14 days; this period corresponds to the half-life of ^{32}P . In this way, the overlap between ^{14}C and ^{32}P channels could be calculated. Radioactivity counts were also corrected for quenching and counting efficiency. An Isocap liquid-scintillation spectrometer was used for radioactivity measurement. The results obtained from triplicate determinations agreed within 5%.

The phosphorus content of phospholipids was determined by the method of Kahovcova & Odavic (1969).

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and Discussion

Incorporation by heart slices of [$Me-^{14}C$]choline and ^{32}P into choline-containing phospholipids

Studies on the formation of sphingomyelin were initiated by incubating heart slices in the presence of both [$Me-^{14}C$]choline and ^{32}P (Table 1). The ratio of specific radioactivities of ^{14}C and ^{32}P in phosphatidylcholine was greater than that in sphingomyelin at all times. Even after 80 min (when the difference between the ratios was less), twice as much labelled phosphorus as labelled choline was incorporated into sphingomyelin. The different labelling pattern between phosphatidylcholine and sphingomyelin, either with [$Me-^{14}C$]choline or ^{32}P , suggested that their synthesis might be unrelated; thus it might not involve a common pool of CDP-choline. The ratio of specific radioactivities of ^{14}C and ^{32}P in phosphatidylcholine decreased with time whereas it remained nearly con-

stant in sphingomyelin. This showed that the equilibration between exogenous and endogenous precursors was different with respect to the sites of synthesis of the two choline-containing phospholipids. This suggested that the syntheses of phosphatidylcholine and sphingomyelin are not only unrelated but also sterically different.

In the next experiments, heart slices were incubated with [^{32}P]P_i for 80 min, then washed and reincubated in isotope-free medium for another 90 min (Fig. 1). In the pulse period, the radioactivity in phosphatidylcholine and sphingomyelin appeared at about the same time, but was slower to accumulate in phosphatidylethanolamine. In the chase period, the labelling of the two choline-containing phospholipids increased further up to about 130 min and then declined in both cases in similar fashion. By contrast, labelling of phosphatidylethanolamine increased until the end of the incubation. This experiment suggested that there was no precursor-product relation

Fig. 1. Kinetics of the incorporation of [^{32}P]P_i into the phospholipids of heart slices

Heart slices (130–140 mg wet wt./ml of bicarbonate buffer) were incubated at 37°C in the presence of [^{32}P]P_i (100 μ Ci/ml). After 80 min (pulse period) the slices were thoroughly washed with ice-cold buffer, and then reincubated in isotope-free medium at 37°C (chase period). At all times, the specific radioactivities of phosphatidylcholine (■), phosphatidylethanolamine (●) and sphingomyelin (▲) were calculated from three independent determinations.

Table 1. *Incorporation of [^{32}P]P_i and [$\text{Me-}^{14}\text{C}$]choline into phosphatidylcholine and sphingomyelin in heart slices* Heart slices (120–130mg wet wt./ml of bicarbonate buffer) were incubated at 37°C in the presence of both [^{32}P]P_i (150 $\mu\text{Ci/ml}$) and [$\text{Me-}^{14}\text{C}$]choline (1 $\mu\text{Ci/ml}$). The average phosphorus contents of the phospholipid spots were as follows: phosphatidylcholine, 6.1 μg ; phosphatidylethanolamine, 5.4 μg ; sphingomyelin, 0.47 μg . The results are the means of three independent determinations.

Time (min)	Specific radioactivity (d.p.m./ μg of P)						$^{14}\text{C}/^{32}\text{P}$ ratio	
	^{14}C		^{32}P					
	Phosphatidyl- choline	Sphingo- myelin	Phosphatidyl- choline	Sphingo- myelin	Phosphatidyl- ethanolamine		Phosphatidyl- choline	Sphingo- myelin
15	345	24	114	39	26		3.02	0.61
30	3220	55	1630	83	65		1.97	0.66
50	5750	104	4090	153	94		1.40	0.67
80	7830	140	6750	236	145		1.16	0.59

between the two choline-containing phospholipids. If a transfer of the phosphorylcholine group from phosphatidylcholine to ceramides was involved in the synthesis of sphingomyelin, then (a) in the pulse period, the appearance of ^{32}P in sphingomyelin should follow that of phosphatidylcholine with a delay, and (b) in the chase period sphingomyelin should continue to incorporate ^{32}P until its specific radioactivity was equal to that of phosphatidylcholine.

With regard to the possibility that different pools of choline were being used for the synthesis of the choline-containing phospholipids, Spitzer *et al.* (1969) reported that with rat liver relatively more [$\text{Me-}^3\text{H}$]choline than [$1,2\text{-}^{14}\text{C}$]choline was incorporated into sphingomyelin compared with phosphatidylcholine. This labelling pattern could only occur if the methyl group of choline serves as a precursor for the synthesis of *S*-adenosylmethionine. This in turn could then methylate ethanolamine, thereby forming new choline moieties, which could subsequently be selectively utilized in the synthesis of sphingomyelin. For such selective utilization, however, the original and new choline moieties should represent different pools. As in the liver, an endogenous pool of choline, derived from the methylation of ethanolamine, may be employed in the synthesis of sphingomyelin in heart tissue. Subsequent experiments were designed to establish the existence of such a pool of choline.

Incorporation of [$1,2\text{-}^{14}\text{C}$]ethanolamine and ^{32}P into phospholipids containing ethanolamine or choline

Instead of [$\text{Me-}^{14}\text{C}$]choline, heart slices were incubated with [$1,2\text{-}^{14}\text{C}$]ethanolamine; in other respects this experiment was identical with that shown in Table 1. The incorporation of ^{14}C radioactivity into the choline moiety of sphingomyelin and phosphatidylcholine showed different patterns (Table 2). Early in

the incubation period they contained ^{14}C radioactivity almost in equal amounts. However, the specific radioactivity of sphingomyelin was twice that of phosphatidylcholine at the end of incubation period. When the choline phospholipids were hydrolysed in 6M-HCl and the hydrolysates analysed by paper chromatography, the ^{14}C radioactivity was identical with the choline spot in both cases. At all times studied, the ratio of the specific radioactivities of ^{14}C and ^{32}P in sphingomyelin was lower than that in phosphatidylethanolamine, but in both cases it remained nearly constant. The same ratio in phosphatidylcholine was at least one order less than that in sphingomyelin and declined rapidly with respect to time. These results indicated the formation of a pool of choline by the methylation of ethanolamine, which was predominantly utilized for the labelling of sphingomyelin.

Labelling of phosphatidylethanolamine by ^{32}P was increased 20-fold by replacing radioactive choline with radioactive ethanolamine. This suggested that the intracellular concentration of ethanolamine was a rate-limiting factor in the synthesis *de novo* of phosphatidylethanolamine. It is particularly noteworthy that the increased labelling of phosphatidylethanolamine by ^{32}P in the presence of ethanolamine was without significant effect on the ^{32}P labelling of sphingomyelin and phosphatidylcholine. This indicated that metabolic interconversions of phospholipids such as (a) methylation of phosphatidylethanolamine to phosphatidylcholine and (b) a subsequent transfer of the phosphorylcholine group from phosphatidylcholine to ceramide, could not be responsible for the appearance of labelled ethanolamine in the choline moiety of sphingomyelin.

A more direct proof that the choline pool that is selectively employed for the synthesis of sphingomyelin was formed by the methylation of ethanolamine is provided below.

Table 2. *Incorporation of [32 P]P_i and [1,2- 14 C]ethanolamine into phospholipids of heart slices*
Heart slices (120–130 mg wet wt./ml of bicarbonate buffer) were incubated at 37°C in the presence of both [32 P]P_i (150 μ Ci/ml) and [1,2- 14 C]-ethanolamine (1 μ Ci/ml). The average phosphorus contents of phospholipid spots were as follows: phosphatidylethanolamine, 5.99 μ g; phosphatidylcholine, 6.88 μ g; sphingomyelin, 0.53 μ g. The results are the means of three independent determinations.

Time (min)	Specific radioactivity (d.p.m./ μ g of P)						$^{14}\text{C}/^{32}\text{P}$ ratio		
	^{14}C			^{32}P					
	Phosphatidyl- ethanolamine	Sphingo- myelin	Phosphatidyl- choline	Phosphatidyl- ethanolamine	Sphingo- myelin	Phosphatidyl- choline			
15	3000	95	112	503	24	157	5.98	3.95	0.71
30	11260	243	205	1890	62	1630	5.95	3.91	0.12
50	15930	571	295	2540	140	4220	6.26	3.93	0.07
80	18030	837	374	3100	247	7190	5.81	3.38	0.05

Labelling of choline-containing phospholipids by S-adenosyl-L-[14 C]methionine

In these experiments, the incorporation of the ^{14}C -labelled methyl groups of S-adenosyl-L-methionine into choline-containing phospholipids in heart was compared with that in other tissues. Thus homogenates from heart, liver, kidney, lung and brain cortex were incubated with S-adenosyl-L-[^{14}C]methionine. The labelling of both phosphatidylcholine and sphingomyelin was linear with time for up to 20 min in heart, liver and kidney homogenates.

Phosphatidylcholine was more highly labelled in liver than in the other tissues, followed by heart and kidney (Table 3). These results agree with published data i.e. that the synthesis of phosphatidylcholine by methylation is a significant pathway in rat liver, but is less important in heart, kidney and lung (Bjornstad & Bremer, 1966) and is practically absent from brain (Ansell & Spanner, 1967). However, heart seems superior to other organs in the rate of incorporation of ^{14}C -labelled methyl groups into sphingomyelin. Also, in comparison with phosphatidylcholine, sphingomyelin was more highly labelled (2.5-fold) in the heart. This value is close to that obtained with labelled ethanolamine (2-fold). These results indicate therefore that radioactive ethanolamine and S-adenosyl-L-methionine labelled the same pool of choline, which then mainly contributed to the formation of sphingomyelin.

In conclusion, the results presented in this paper strongly suggest that a pool of choline exists in the rat heart that is selectively used for the synthesis of sphingomyelin. This may mean that the intracellular localization of the synthesis of sphingomyelin is dif-

Table 3. *Incorporation of S-adenosyl-L-[14 C]methionine into the phospholipids of rat organs*

The standard incubation mixture contained 17.6 nM-S-adenosyl-L-[^{14}C]methionine, 50 mM-Tris/HCl buffer, pH 7.4, and portions of homogenates of heart, liver, brain cortex, kidney or lung (2.1–2.9 mg of protein), in a final volume of 0.5 ml. Mixtures were incubated in a shaking water bath for 20 min at 37°C. The results are the means of four independent determinations.

Organ (homogenates)	Specific radioactivity (d.p.m./ μ g of P)	
	Phosphatidyl- choline	Sphingomyelin
Heart	9560	23900
Liver	80400	7690
Brain cortex	112	1000
Kidney	1720	2250
Lung	287	657

ferent from that of phosphatidylcholine in the heart. The nature of this pool of choline, however, is not clear at present. The results presented here support the view that this pool of choline is formed by the methylation of non-esterified ethanolamine. Recently, the methylation of free ethanolamine in the brain tissue has been described (Kewitz & Pleul, 1976), but results with regard to the heart are lacking.

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