Sphingomyelin and ceramide-phosphoethanolamine synthesis by microsomes and plasma membranes from rat liver and brain

Monique Malgat, Andrée Maurice, and Jacques Baraud

Laboratoire de Biochimie, Université de Bordeaux II, 146 rue Léo Saignat, 33076 Bordeaux cedex, France

Abstract Pulse-chase experiments showed that phosphatidylethanolamine (PE) was the direct precursor for ceramide-phosphoethanolamine, a sphingomyelin analogue, in the same way as phosphatidylcholine was for sphingomyelin. Ceramide-phosphoethanolamine could be identified by incorporation of radioactivity from labeled PE, as well as by its stability in alkaline methanolysis and its ability to be methylated by S-adenosyl-methionine. Ceramide-phosphoethanolamine synthesis from labeled exogenous PE seemed to be independent of exogenous ceramide; it was proportional to the amount of incubated membrane, when taking into account the isotopic dilution of labeled precursor by endogenous PE. Sphingomyelin synthesis, which was demonstrated using natural PC as a substrate, was not possible using dipalmitoyl-PC. The formation of sphingomyelin and ceramide-phosphoethanolamine was demonstrated in microsomes and plasma membranes from rat brain and liver. —**Malgat, M., A. Maurice, and J. Baraud.** Sphingomyelin and ceramide-phosphoethanolamine synthesis by microsomes and plasma membranes from rat liver and brain. *J. Lipid Res.* 1986. **27:** 251–260.

Supplementary key words phosphatidylcholine • phosphatidylethanolamine • biosynthesis

In sphingomyelin biosynthesis, phosphatidylcholine (PC) is known as the proximal donor of phosphocholine transferred to ceramide (1–4). The enzyme catalyzing this reaction is a transferase, not a hydrolase (4). Phospholipid exchange proteins facilitate the introduction of exogenous PC into the membrane containing PC:ceramide-cholinephosphotransferase (5). This enzyme was found in microsomes from mouse liver (1), monkey liver and heart (2), in plasma membrane and Golgi fractions from cultured mouse fibroblasts (3), in plasma membrane from cultured hamster kidney cells (4) and from mouse Ehrlich ascites (5).

Mouse brain microsomes did not produce sphingomyelin when incubated with labeled ceramide, but rather produced a sphingomyelin analogue in which ethanolamine took the place of choline (1, 2).

Previous work showed that sphingomyelin was synthesized by a single membrane, either microsomes or plasma membrane, according to the cells that were studied, and that this membrane produced a single sphingomyelin class, i.e., sphingomyelin itself. Brain microsomes, however, were found to produce ceramide-phosphoethanolamine.

In this study, we attempted to demonstrate the three following points: *1*) sphingomyelin is produced by both microsomes and plasma membrane from rat liver; *2*) rat brain microsomes can synthesize ceramide-phosphoethanolamine as well as sphingomyelin, and both can also be synthesized by rat brain plasma membrane; and *3*) rat liver microsomes and plasma membrane may produce ceramide-phosphoethanolamine as well as sphingomyelin, when PE is used as precursor instead of PC.

MATERIALS AND METHODS

Materials

Cytidyl-5'-diphospho[1,2-^{14}C]ethan-1-ol-2-amine was obtained from ICN; cytidine 5'-diphospho[methyl-^{14}C]-choline dipalmitoyl, 1,2-dioleoyl-*sn*-glycerophospho[2-^{14}C]-ethanolamine, and S-adenosyl[methyl-^{3}H]methionine were purchased from Amersham-France. Unlabeled products were from Sigma Chemical Co.

Isolation of subcellular membranes

Subcellular membranes were derived from liver and brain of Wistar rats (250 g).

Plasma membrane fractions and microsomes were obtained from a liver homogenate, 20% in 15 mM Tris-HCl, 1 mM $MgCl_2$, 0.25 M sucrose, pH 7.4, by a modification of the procedure of Hashizume et al. (6). Unbroken cells and nuclei were discarded, and the homogenate was centrifuged for 10 min at 10,000 *g*. The resulting super-

Abbreviations: DOPE, Dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; TLC, thin-layer chromatography.

natant was centrifuged for 20 min at 15,000 *g*. The pellet was discarded and the supernatant was centrifuged again for 120 min at 100,000 *g*; the microsomal pellet was washed by recentrifugation. The 10,000-*g* pellet was suspended in 10% (w/w) sucrose and loaded onto a 10%, 30%, 50% discontinuous sucrose gradient. Centrifugation was carried out for 120 min at 100,000 *g*. Plasma membrane fractions were collected at the 30–50% interfacial band. These fractions were resuspended in pH 7.4 buffer, collected, and washed by centrifugation for 60 min at 100,000 *g*.

Synaptic membrane fractions and microsomes were obtained from brain homogenate according to Roth, Laskowski, and Coscia (7).

All the fractions were lyophilized and stored at −20°C.

Enzyme assays

NADPH-cytochrome *c*-reductase activity was determined (8) as a marker enzyme for microsomal fractions, and 5'-nucleotidase (9) was determined for plasma and synaptic membrane fractions. There was practically no contamination of microsomes by plasma or synaptic membranes. The contamination of plasma or synaptic membrane fractions by microsomes, which is difficult to avoid, was always less than 7%.

Protein determination

The protein content of each membrane fraction was determined by the method of Lowry et al. (10), using bovine serum albumin as standard.

Extraction and study of lipids

Extraction. Total lipids were extracted with 2 ml of chloroform–methanol 2:1 and washed with 50 mM KCl. The final chloroform phase was dried under a stream of nitrogen and diluted in a known volume. One aliquot was used for radioactivity determination and another for phospholipid separation.

Chromatography. Phospholipids were separated by one-dimensional TLC on silica gel G plates (Merck). The solvent system was chloroform–methanol–ammonia–water 72:48:2:9 (v/v) or, for double development, (A) chloroform–methanol–acetic acid–water 50:25:8:2.5 (v/v) and (B) chloroform–methanol–ammonia 65:35:8 (v/v). Iodine staining was used for visualization of the compounds.

Determination of radioactivity. All samples were counted in a Packard Tricarb Scintillation Spectrometer, either in a 10-ml toluene system, or in a 10-ml dioxan system when radioactivities of water-soluble and lipid-soluble products were to be compared.

Alkaline methanolysis. Whatever the solvent system used, it was difficult to quantitatively separate sphingomyelin or ceramide-phosphoethanolamine from lyso-PC or lyso-PE. Therefore, the lipid extract was subjected to mild alkaline methanolysis in 0.3 N NaOH–methanol at 37°C for 1 hr. Then the solution was cooled and neutralized by HCl. The chloroform-extractable products (sphingomyelin or its analogue) were recovered and carefully washed with 50 mM KCl. All other lipids, especially PC, PE, and lyso-compounds, were converted into water-soluble compounds, i.e., glycerophosphocholine and glycerophosphoethanolamine. When analyzed by TLC, sphingomyelin or ceramide-phosphoethanolamine gave a single spot, and were identified by their R_f values: 5.4 for sphingomyelin and 6.0 for its analogue, respectively.

Labeled PC synthesis

Liver microsomes (700 μg of protein) were incubated with 31 μM CDP-[^{14}C]choline (57 mCi/mmol) + 3.1 mM diacylglycerol for 3 hr at 37°C. The reaction was stopped by adding 2 ml of chloroform–methanol 2:1. Total lipids in chloroform were washed and separated by TLC. PC was eluted from the silica gel with chloroform–methanol 1:2. Its specific activity was estimated by measuring the radioactivity and lipid phosphorus, according to Napias (11).

In vitro assays of sphingomyelin synthesis

Incubations. Membrane fractions (250–350 μg of protein) were suspended in 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 0.15 mM KCl. Labeled PC or PE, and sometimes ceramide, were sonicated in the same buffer, in the presence of Triton X-100 (20 μg/ml of incubation medium). Reactions were performed in a final volume of 350 μl. After 3 hr at 37°C, the assays were stopped by addition of 2 ml of chloroform–methanol 2:1. The precursor concentrations varied according to the assays and will be specified for each experiment. Lipids were extracted and studied as described above.

Pulse-chase: Assay I. Liver microsomal fractions (650 μg of protein) were incubated in 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 0.15 mM KCl, with 31 μM radiolabeled cytidyl-5'-diphospho[methyl-^{14}C]choline (57 mCi/mmol) and 3.1 mM diacylglycerol sonicated with Triton X-100 (20 μg/ml of incubation medium); total volume, 550 μl. After incubation for 120 min at 37°C, 100 μl was removed and the reaction was stopped by adding 2 ml of chloroform–methanol 2:1. The chase period was immediately started with the remainder by adding 40 μl of 10 mM unlabeled CDP-choline. Every other hour for 8 hr, 120 μl was removed and the reaction was stopped as previously described. Lipids were extracted and studied as described above. *Assay II.* The reaction medium (550 μl) contained liver microsomal fraction (650 μg of protein), 47 mM cytidyl-5'-diphospho[1,2-^{14}C]ethanolamine (97 mCi/mmol), and 4.6 mM diacylglycerol, sonicated as above. For the chase period, 50 μl of 11.2 mM unlabeled CDP-ethanol-

amine was added. *Assay III* was like assay II, with a brain microsomal fraction.

Methylation

Radiolabeled ceramide-phosphoethanolamine was obtained as indicated above, by incubating brain synaptic membrane with *sn*-phosphatidyl-[2-^{14}C]ethanolamine (97 mCi/mmol). PE and other phospholipids in excess were eliminated by alkaline methanolysis, and purified ceramide-phosphoethanolamine was recovered as indicated above. It was sonicated in 15 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.25 M sucrose, EDTA 0.1 mM, containing Triton X-100 (20 μg/ml of final incubation medium). An aliquot was incubated, in the same buffer, with the membrane fraction and *S*-adenosyl-[methyl-^{3}H]methionine (500 mCi/mmol); the total volume was 270 μl. The methylation reaction was performed at 37°C for 4 hr. After extraction, phospholipids were carefully washed with 50 mM KCl until the aqueous phase became unlabeled. Membrane phospholipids were eliminated by a second alkaline methanolysis. Ceramide-phosphoethanolamine and sphingomyelin remaining in the chloroform phase were estimated by measuring ^{14}C and ^{3}H radioactivity. During this time, the membrane fraction was incubated with labeled S-adenosylmethionine, without labeled ceramide-phosphoethanolamine, in order to estimate the methylation of ceramide-phosphoethanolamine produced from endogenous PE during the methylation time. In fact, methylation in this control study was not significant.

RESULTS

Sphingomyelin precursors

Pulse-chase experiments were performed in order to demonstrate that PC and PE were direct precursors for sphingomyelin and ceramide-phosphoethanolamine.

Liver microsomes were incubated with CDP-[^{14}C]choline or CDP-[^{14}C]ethanolamine and diacylglycerol. After PC or PE labeling for 2 hr at 37°C, a large excess of unlabeled CDP-choline or CDP-ethanolamine was added. An aliquot was removed every other hour for 8 hr. For each sample, the reaction was stopped by adding 2 ml of chloroform–methanol 2:1. The lipid extract was washed and subjected to alkaline methanolysis. Sphingomyelin or ceramide-phosphoethanolamine was extracted with $CHCl_3$ after addition of 50 mM KCl. Water-soluble products from methanolysis of PC or PE remained in the aqueous phase. Radioactivity of both phases was measured (**Fig. 1**).

[^{14}C]Choline and [^{14}C]ethanolamine were readily incorporated into PC and PE during the 2-hr labeling period; however, during this time, the rate of labeling incorporation into sphingomyelin and ceramide-phosphoethanolamine was practically insignificant. The chase-period

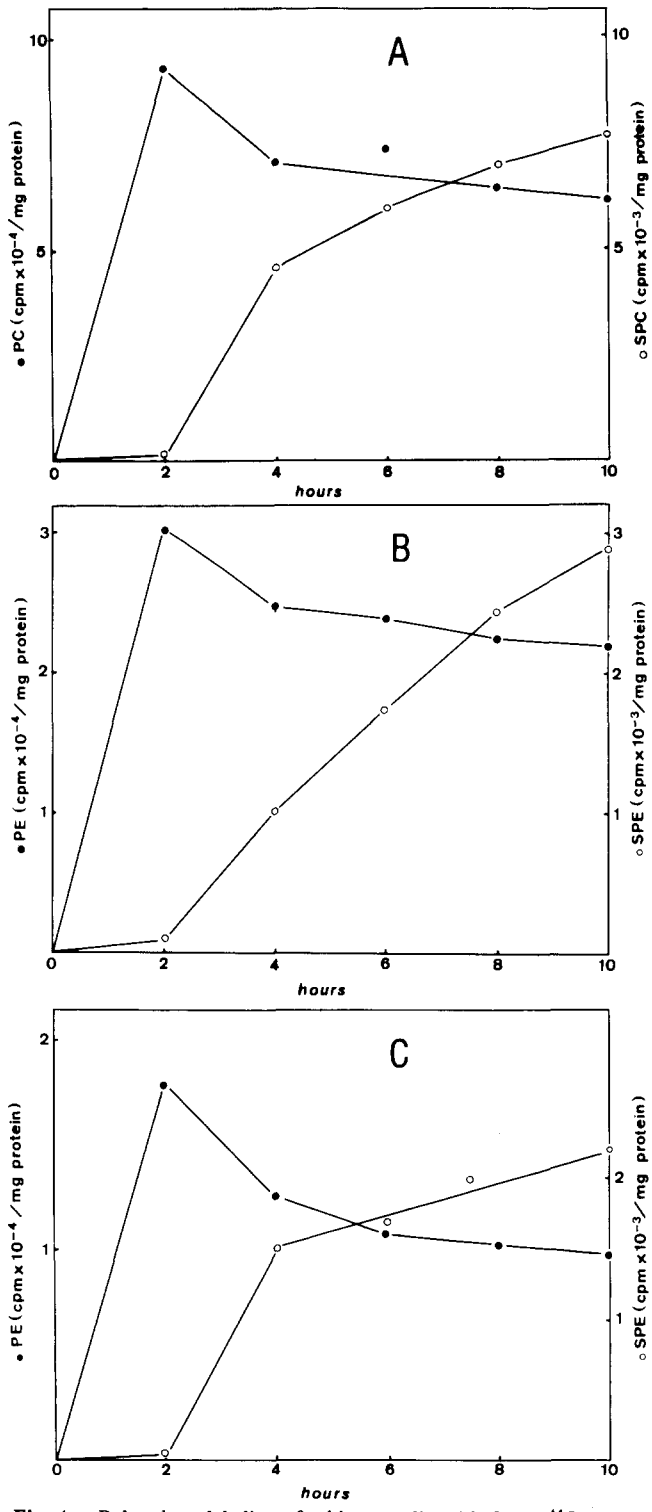

Fig. 1. Pulse-chase labeling of sphingomyelin with CDP-[^{14}C]choline or CDP-[^{14}C]ethanolamine. A, Liver microsomes (650 μg protein) were incubated with 31 μM CDP-[^{14}C]choline (57 mCi/mmol) + 3.1 mM diacylglycerol in a final volume of 550 μl. B, Liver microsomes (650 μg protein) were incubated with 47 μM CDP-[^{14}C]ethanolamine (97 mCi/mmol) + 4.6 mM diacylglycerol. The final volume was 550 μl. C, Brain microsomes (650 μg protein) were incubated as in B. In all cases, an aliquot was removed after 2 hr and the chase period was initiated by adding (A) 40 μl of 10 mM unlabeled CDP-choline, (B) and (C), 50 μl of 11.2 mM unlabeled CDP-ethanolamine; SPC, sphingomyelin; SPE, ceramide-phosphoethanolamine.

showed a decrease in activity of both PC and PE. In contrast, the incorporation of labeled choline and ethanolamine into sphingomyelin and ceramide-phosphoethanolamine proceeded at nearly a linear rate for at least 8 hr.

These results implied that sphingomyelin and ceramide-phosphoethanolamine syntheses were directly dependent upon the transfer of phosphocholine from PC or phosphoethanolamine from PE, and not from CDP-choline or CDP-ethanolamine. The results were in agreement with those obtained by Voelker and Kennedy (4) for pulse-chase labeling of PC and sphingomyelin with [methyl-^{3}H]-choline in BHK 21 cells. In addition, our experiments showed that endogenous PE could be the direct precursor of ceramide-phosphoethanolamine.

A similar result was obtained by pulse-chase experiments with brain microsomes incubated with CDP-[^{14}C]-ethanolamine (Fig. 1C).

Cellular localization of the biosynthesis

Sphingomyelin and ceramide-phosphoethanolamine synthesis was studied with four membrane fractions: rat liver microsomes and plasma membrane, rat brain microsomes and synaptic membrane. Three precursors were used: [^{14}C]choline-labeled DPPC, [^{14}C]choline-labeled PC obtained by incubation of rat liver microsomes with CDP-[^{14}C]choline (see Materials and Methods), and [^{14}C]ethanolamine-labeled DOPE.

Incubation with DPPC. Membrane fractions were incubated with [^{14}C]choline-labeled DPPC for 3 hr at 37°C, pH 7.4. The lipid extract was subjected to mild alkaline methanolysis, as previously described, so that the residual DPPC was converted into water-soluble products, while sphingomyelin was extracted with chloroform. The radioactivity of both phases was measured and the PC:ceramide-cholinephosphotransferase activity was expressed as the amount of labeled sphingomyelin (nmol/mg membrane protein per hr).

Table 1 shows that for the two DPPC concentrations in incubations at 37°C, there was practically no transfer of phospho[^{14}C]choline into sphingomyelin. Some standard deviations were nearly as high as the values, due to the difficulty in recovering the very small amount of radioactivity in many of the experiments. Measured activities were at the limit of significant detection. It seemed likely that, since the incubation temperature was below the DPPC transition temperature (42°C), the molecular rigidity did not allow DPPC to interact with PC:ceramide-phosphocholinetransferase. Assays at 46°C showed that this hypothesis was incorrect, since the rate of synthesized sphingomyelin was not increased, even in the presence of exogenous *erythro*-ceramide, although the cholinephosphotransferase activity could be reduced at 46°C (5).

It is possible that the enzyme cannot interact with a PL in which both fatty acids are saturated. DPPC is representative of membrane PC, but there was nothing to suggest that it could be a precursor for sphingomyelin synthesis. Probably, for this reason, previous investigators used natural PC synthesized from choline or CDP-choline by cells or microsomes.

Incubation with labeled microsomal PC. Labeled natural PC, used as precursor, was obtained by incubation of rat liver microsomes with CDP-[^{14}C]choline, as described in Materials and Methods. It might have contained some glycerylether-PC. When submitted to alkaline methanolysis, these products lead to lyso-glycerylether-PC which could not be separated from sphingomyelin by TLC, as described in Materials and Methods. Therefore, after incubation of labeled PC with microsomes, the lipid extract was subjected to high performance TLC, according to Heape et al. (12). The solvent system was methyl acetate–n-propanol–chloroform–methanol–0.25% aqueous KCl 25:25:28:10:7 (v/v). By this new technique, lyso-PC, sphingomyelin, and PC + glycerylether-PC were separated, with R_f values of 3.1, 5.4, and 9.9, respectively. The fraction PC + glycerylether-PC was submitted to alkaline methanolysis; PC was converted into water-soluble by-products, while glycerylether-PC remained extractable

TABLE 1. Incorporation of phosphocholine from exogenous PC into sphingomyelin

	Specific Activity[a]					
	Microsomal PC at 37°C		DPPC at 37°C		280 μM DPPC at 46°C	
Membrane Fractions	200 μM	400 μM	140 μM	280 μM	− Ceramide	+ Ceramide
Liver						
Microsomes	2.5 ± 0.3	3.6 ± 0.4	0.10 ± 0.05	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Plasma membrane	2.1 ± 0.2	2.8 ± 0.2	0.15 ± 0.05	0.1 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
Brain						
Microsomes	3.0 ± 0.3	4.4 ± 0.4	0.05 ± 0.05	0.3 ± 0.1	0.35 ± 0.1	0.1 ± 0.1
Synaptic membrane	5.2 ± 0.4	7.6 ± 0.5	0.10 ± 0.05	0.2 ± 0.1	0.35 ± 0.1	0.25 ± 0.1

Membrane fractions (250 μg protein) were incubated with 50 or 100 nmol of [^{14}C]choline-labeled DPPC (2.7 mCi/mmol) or with 70 or 140 nmol of microsomal PC (1660 cpm/nmol) for 3 hr at 37°C or 46°C. In one case, 95 nmol of *erythro*-ceramide was added. The final volume was 350 μl. The lipids were extracted and subjected to alkaline methanolysis. The radioactivity of sphingomyelin and residual PC was estimated as described in Materials and Methods. Values represent means of three determinations conducted with different membrane preparations.

[a] (nmol sphingomyelin/mg protein per hr) of cholinephosphotransferase based on sphingomyelin formation from exogenous PC.

by chloroform. This extract did not exhibit measurable radioactivity. This result was not surprising, since the amount of glycerylether-PL is only 0.8% of total liver lipids (13) and is mainly comprised of glycerylether-PE.

Table 1 shows that when labeled natural PC was used as precursor, labeled phosphocholine was incorporated into sphingomyelin by the four membrane fractions. Incorporation by brain and liver microsomal fractions was comparable, but much higher for brain synaptic membrane than for liver plasma membrane. These results confirmed our hypothesis that PC could contain an unsaturated fatty acid to function as a substrate for PC:ceramide-cholinephosphotransferase.

The results presented in Table 1 are underestimated, since endogenous PC is expected to dilute the specific activity of the labeled precursor. This factor will be discussed below.

Incubation with DOPE. Membrane fractions were incubated with [^{14}C]ethanolamine-labeled DOPE for 3 hr at 37°C. In some cases, 5 mM beta-hydroxyethylhydrazine was added to prevent any methylation of PE or ceramide-phosphoethanolamine into PC or sphingomyelin, respectively (14). **Table 2** shows that results obtained with DOPE were similar to those obtained with microsomal PC, i.e., the four membrane fractions were able to synthesize ceramide-phosphoethanolamine from PE. This product was identified by incorporation of PE radioactivity, even in the presence of methylation inhibitor, as well as by its stability to alkaline methanolysis and its capacity to be methylated, as demonstrated below.

Alkaline methanolysis, which hydrolyzed glycerophospholipids into water-soluble by-products, did not destroy alkenyl-glycerophospholipids (plasmalogens) or alkylglycerophospholipids, which were only converted into lyso-compounds. These glycerylether-PL, especially plasmalogens, were found in relatively large amounts in membranes, particularly in brain membranes, and included ethanolamine glycerophospholipids to a large degree. In order to demonstrate that they did not interact with sphingomyelin or ceramide-phosphoethanolamine estimations, microsomes or plasma membranes were incubated with labeled DOPE and the lipid extracts were analyzed by TLC. The PE fraction, also containing glycerylether-PE, was separated and subjected to alkaline methanolysis. Chloroform-extractable products (lysoglycerylether-PE) were devoid of radioactivity, thus showing that the phosphoethanolamine moiety was not derived from PE. This is consistent with the mechanism of glycerylether-PL biosynthesis (13), i.e., phosphoethanolamine is provided by CDP-ethanolamine, as in PE biosynthesis. Subsequently, our incubation experiments suggested that PE was a donor of phosphoethanolamine only in ceramide-phosphoethanolamine synthesis.

TABLE 2. Incorporation of phosphoethanolamine from exogenous PE into ceramide-phosphoethanolamine

Membrane Fractions	Specific Activity[a] – Ceramide	+ Ceramide	+ Ceramide, with Correction of Isotopic Dilution
Liver			
Microsomes	2.5 ± 0.2[b]	2.4 ± 0.2	2.8 ± 0.3
Plasma membrane	3.4 ± 0.4	3.1 ± 0.1	4.1 ± 0.2
Brain			
Microsomes	4.4 ± 0.3	4.7 ± 0.3	5.8 ± 0.4
Synaptic membrane	6.6 ± 0.4	6.6 ± 0.3	9.35 ± 0.5

Membrane fractions (250 μg of protein) were incubated with 132 nmol of [^{14}C]ethanolamine-labeled DOPE (0.92 mCi/mmol) with or without 190 nmol of *erythro*-ceramide for 3 hr at 37°C. The final volume was 350 μl. The lipids were extracted and subjected to alkaline methanolysis. Radioactivity of ceramide-phosphoethanolamine and residual DOPE was estimated as described in Materials and Methods.

[a] (nmol ceramide-phosphoethanolamine/mg protein per hr) of ethanolaminephosphotransferase based on ceramide-phosphoethanolamine formation from exogenous PE.

[b] Values represent means of five determinations.

The results presented in Table 2 are underestimated owing to the isotopic dilution of labeled DOPE by membrane endogenous PE. By taking into account the isotopic dilution, a better comparison of the specific activity of the PE:ceramide-ethanolaminephosphotransferase can be seen in the four membranes.

Ceramide-phosphoethanolamine synthesis from labeled DOPE appeared to be independent of added ceramide (Table 2). Voelker and Kennedy (4) have shown that exogenous ceramide was not incorporated into sphingomyelin when added to incubations of plasma membranes of BHK 21 cells with exogenous PC.

Varying amounts of [^{14}C]ethanolamine-labeled DOPE were incubated with a constant amount of membrane fraction, i.e., constant concentration of both endogenous ceramide and PE:ceramide-ethanolaminephosphotransferase. It can be seen for the four membrane fractions that the amount of ceramide-phosphoethanolamine synthesized in each case was constant from a PE concentration of 200–250 μM (**Fig. 2**). These experiments suggested that the endogenous ceramide concentration could be a limiting factor, as could the amount of PE:ceramide-ethanolaminephosphotransferase.

It should be noted that the reaction rate, as in all our incubations, was linear at least for 4 hr with an excess of exogenous PE (**Fig. 3**), even up to 8 hr (5).

In other experiments, different amounts of brain synaptic membrane (from 50 μg to 1 mg of protein) were incubated with a constant concentration of [^{14}C]ethanolamine-labeled DOPE. **Table 3** shows that when the amount of incubated membrane was increased the radioactivity incorporated into ceramide-phosphoethanolamine from DOPE increased at the same time. This is consistent with a higher concentration of both endogenous ceramide and PE:ceramide-ethanolaminephosphotransferase. However,

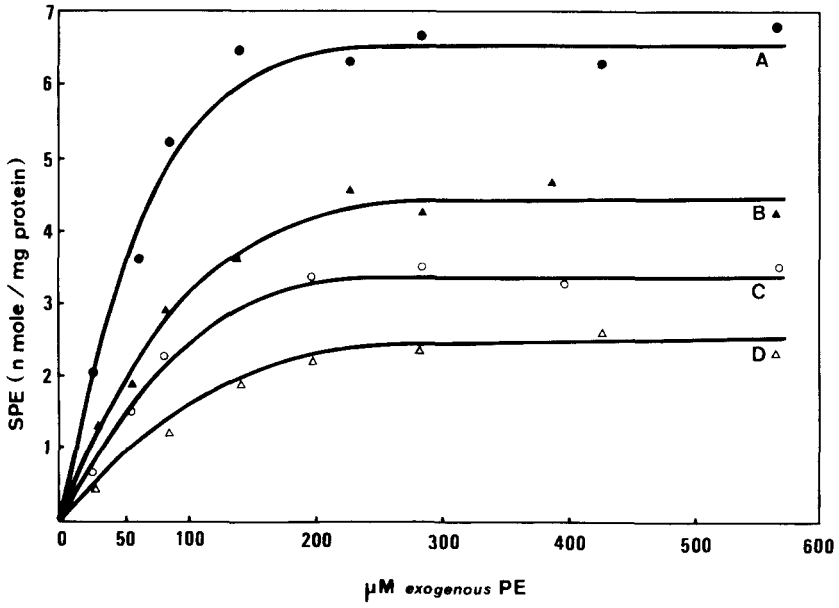

Fig. 2. Dependence of ceramide-phosphoethanolamine upon the amount of exogenous PE. A constant amount of membrane fraction (250 μg protein) was incubated with different amounts of [^{14}C]ethanolamine-labeled DOPE (97 mCi/mmol) for 3 hr at 37°C. The final volume was 350 μl. Ceramide-phosphoethanolamine (SPE) is expressed as nmol/mg protein. Points plotted are averages of three determinations. A, Brain synaptic membrane; B, brain microsomes; C, liver plasma membrane; D, liver microsomes.

the increase was not proportional because labeled PE was diluted by endogenous PE. We found that, for brain synaptic membrane, the PL/protein ratio was 0.70 and endogenous PE was 25% of total PL. In fact, endogenous PE was estimated to be 22 nmol per 100 μg of membrane protein. This isotopic dilution taken into account, the amount of ceramide-phosphoethanolamine synthesized was proportional to the amount of incubated membrane. All values, calculated for 1 mg of protein, approximated 9 nmol. These results could indicate that exogenous and endogenous PE constitute a single pool for ceramide-phosphoethanolamine synthesis.

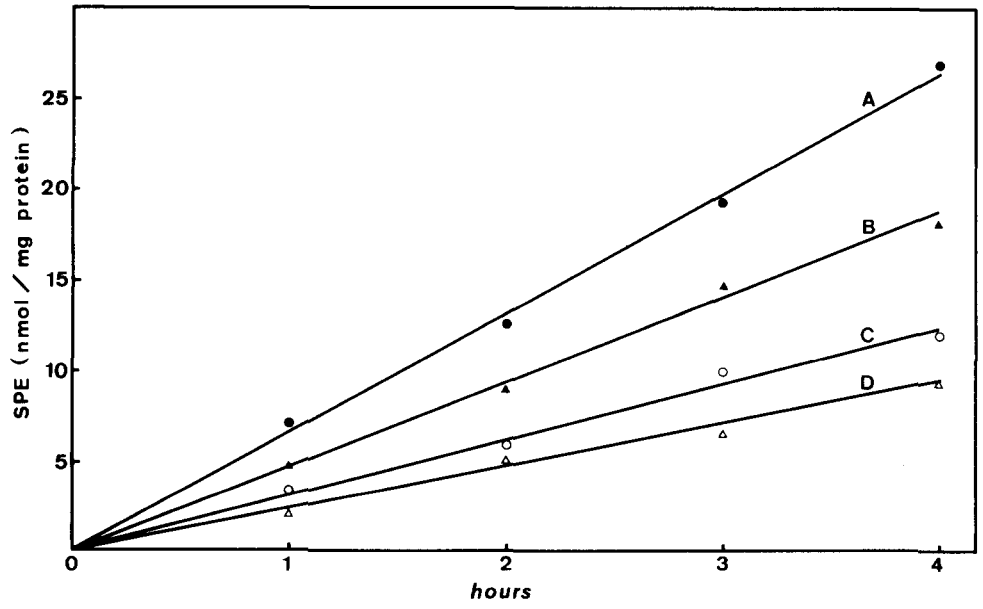

Fig. 3. Time course of ceramide-phosphoethanolamine synthesis from exogenous PE. See conditions in Table 2, except incubation time, which was variable from 1 to 4 hr; SPE, ceramide-phosphoethanolamine. A, Brain synaptic membrane; B, brain microsomes; C, liver plasma membrane; D, liver microsomes.

TABLE 3. Dependence of ceramide-phosphoethanolamine formation from labeled DOPE upon the concentration of brain synaptic membrane

Membrane	Incorporated Radioactivity	Ceramide-Phosphoethanolamine Synthesized from A Exogenous PE	B Exogenous + Endogenous PE	C as B, Expressed for 1 mg Protein
μg protein	*cpm*		*nmol*	
50	1605	0.45	0.5	10.0
100	2500	0.7	0.85	8.4
250	5640	1.6	2.3	9.2
350	7015	2.0	3.1	8.8
500	8325	2.4	4.4	8.8
1000	11820	3.4	9.1	9.1

Different amounts of brain synaptic membrane were incubated with a constant concentration (132 nmol) of [^{14}C]ethanolamine-labeled DOPE (0.92 mCi/mmol) for 3 hr at 37°C. The final volume was 350 μl. Ceramide-phosphoethanolamine was extracted and purified by alkaline methanolysis, and its radioactivity was estimated as described in Materials and Methods.

Methylation of ceramide-phosphoethanolamine

Labeled ceramide-phosphoethanolamine was obtained after incubation of membrane fractions with [^{14}C]ethanolamine-labeled PE and purification after alkaline methanolysis. An aliquot was incubated with a membrane fraction (50 μg of protein) and S-adenosyl-[methyl-^{3}H]methionine for 4 hr at 37°C, pH 7.4. It was important to use only 50 μg of membrane protein, in order to limit the utilization of *S*-adenosylmethionine for endogenous PE methylation, and to limit the conversion of endogenous PE into ceramide-phosphoethanolamine during the methylation period. A control without exogenous ceramide-phosphoethanolamine was performed in order to estimate the methylation of ceramide-phosphoethanolamine produced from endogenous PE.

Ceramide-phosphocholine produced by this reaction was labeled both by ^{14}C and ^{3}H. It was recovered with the unmethylated ceramide-phosphoethanolamine, purified as described in Materials and Methods, and estimated by measuring ^{14}C and ^{3}H radioactivity.

It was not possible to calculate the ^{3}H/^{14}C ratio in sphingomyelin since it was not separated from its precursor. Sphingomyelin was estimated by its ^{3}H-radioactivity; in our experiments, each nmol of $-CH_3$ group incorporated had a radioactivity of 368,000 cpm (after correction of ^{14}C interference); three $-CH_3$ groups were required for each mole of sphingomyelin.

The results are expressed (**Table 4**) as the percentage of ceramide-phosphoethanolamine methylated per 1 mg of protein for 4 hr. They showed that the methylation rate was higher in liver than in brain, but nearly equivalent for the two membrane fractions of each organ.

Sphingomyelin from ceramide-phosphoethanolamine could be obtained by direct methylation as well as from the following reactions: ceramide-phosphoethanolamine → PE → PC → sphingomyelin. Two experiments were performed in order to solve this problem.

1) Labeled ceramide-phosphoethanolamine (10 nmol), obtained as previously described, was incubated with a membrane fraction (50 μg of protein) with or without an excess of diacylglycerol (100 nmol). The reaction was stopped after 4 hr and the lipids were extracted and submitted to alkaline methanolysis. In the absence of exogenous diacylglycerol, the amount (nmol) of ceramide-phosphoethanolamine converted into PE/mg protein per hr, was 0.14 for liver microsomes, 0.18 for brain microsomes, 0.28 for liver plasma membrane, and 0.25 for brain synaptic membrane, respectively. In the presence of exogenous diacylglycerol, the results were about the same, showing that exogenous diacylglycerol was no more utilizable than exogenous ceramide in the reverse reaction.

2) Membrane fractions (50 μg of protein) were incubated with 20 nmol of S-adenosyl-[^{3}H]methionine at 37°C for 4 hr. After reaction, the lipids were extracted and separated by TLC and PC radioactivity was measured. The amount of PE (pmol) converted into PC, per mg of membrane protein, was 70 for liver microsomes, 5.5 for liver plasma membrane, 66 for brain microsomes, and 13 for brain synaptic membrane, respectively. These very slight methylation amounts were not surprising since the substrate was endogenous PE and not an excess of exogenous PE, which would change the PE/PC ratio. A similar result was obtained (14) with microsomes from rat aorta.

These results are not a direct demonstration of the conversion of ceramide-phosphoethanolamine to sphingomyelin; but, since both the reaction ceramide-phosphoethanolamine → PE and the methylation of endogenous PE occurred to a very low degree, it seems reasonable to assume that sphingomyelin was obtained from ceramide-phosphoethanolamine by direct methylation. For instance, in the case of liver microsomes (50 μg of protein), 3 nmol of ceramide-phosphoethanolamine would yield 0.055 nmol of PE, which would be submitted to an isotopic dilution by 4.6 nmol of endogenous PE. Then only 70 pmol would be methylated into PC and diluted again by 15 nmol of endogenous PC. Under these conditions, it would not be possible to obtain 0.28 nmol of ^{3}H-labeled sphingo-

TABLE 4. Ceramide-phosphoethanolamine methylation by S-adenosyl-[methyl-^{3}H]-methionine in the presence of various membrane fractions

Membrane fractions	Radioactivity			Methylated Ceramide-Phosphoethanolamine
	^{14}C Recovered after Reaction		^{3}H Incorporated into Sphingomyelin	
	cpm	%	*cpm*	*nmol per 100/mg protein · 4 hr*
Liver				
Microsomes	18,360	86	15,455 ± 1,830	9.3 ± 1.1
Plasma membrane	18,300	86	14,620 ± 1,660	8.8 ± 1.0
Brain				
Microsomes	18,800	88	4,025 ± 840	2.4 ± 0.5
Synaptic membrane	17,950	84	1,810 ± 490	1.1 ± 0.3

Ceramide-phosphoethanolamine was obtained by incubation of brain synaptic membrane with [^{14}C]ethanolamine-labeled DOPE as described in Table 2. It was purified by alkaline methanolysis. An aliquot (3 nmol; total ^{14}C radioactivity, 21,350 cpm) was incubated with membrane fraction (50 μg of protein) and 50 nmol of S-adenosyl-[methyl-^{3}H]methionine (500 mCi/mmol) in a total volume of 270 μl, for 4 hr at 37°C, pH 7.4. Sphingomyelin and ceramide-phosphoethanolamine were recovered, purified as described in Materials and Methods, and estimated by measure of ^{14}C and ^{3}H radioactivity. The results are means of three assays.

myelin. The recovery of 84–88% ^{14}C-radioactivity was compatible with a very small amount of substrate converted into PE or PC and then destroyed by alkaline methanolysis.

It can be concluded that the four membrane fractions contain N-methyltransferases that catalyze a direct methylation of ceramide-phosphoethanolamine to form sphingomyelin.

DISCUSSION

Sphingomyelin is known to be synthesized by a PC:ceramide-phosphocholinetransferase which translocates phosphocholine from PC to ceramide. According to Voelker and Kennedy (15), this translocation is facilitated by an exchange protein when the phosphocholine donor is exogenous PC.

In this study, we showed that PC is the direct precursor for sphingomyelin in rat liver microsomes. This is in agreement with studies of Ullman and Radin (1) and Bernert and Ullman (2). Table 1 shows that the same result was obtained with a similar rate in rat liver plasma membranes, and can be compared to the results obtained with other plasma membranes—from fibroblasts by Marggraf et al. (3), from cultured hamster kidney cells BHK 21 by Voelker and Kennedy (4), and from mouse Ehrlich ascites by Marggraf and Kanfer (5). The microsomal synthesis was demonstrated by Lipsky and Pagano (16) who observed that, in hamster lung fibroblasts, endogenously synthesized fluorescent sphingomyelin is translocated "through the Golgi apparatus en route to the plasma membrane." But they did not observe any synthesis in plasma membrane—its sphingomyelin would be provided by membrane flow.

Nevertheless, our results were positive only with "natural PC," obtained by incubation of liver microsomes with CDP-[^{14}C]choline. On the other hand, DPPC could not be used as a phosphocholine donor. It was originally thought that incubation at 37°C, below the transition temperature (42°C), was responsible for this negative result, but incubations at 46°C led to the same result. It could also mean that PC must contain an unsaturated fatty acid to be used as substrate by PC:ceramide-cholinephosphotransferase.

Similar results were obtained for the first time by incubation of microsomes and synaptic membrane from rat brain. With DPPC, the sphingomyelin synthesis was insignificant, but, with natural PC, the rate of synthesis per mg of protein was higher for brain membrane fractions than for liver, especially for synaptic membrane.

We have described the synthesis of ceramide-phosphoethanolamine, a sphingomyelin analogue, from exogenous PE in liver microsomes and plasma membrane. This product was obtained (1, 2) only from ceramide and endogenous PE of mouse brain microsomes. We have demonstrated (Table 2) that its synthesis was possible from exogenous PE, not only in brain microsomes, but also, more importantly, in synaptic membrane.

Ceramide-phosphoethanolamine was identified by several procedures: i) by TLC, its R_f was slightly different from that of sphingomyelin, but not different enough to allow a quantitative separation; ii) by incorporation of radioactivity from [^{14}C]ethanolamine-labeled PE, even in the presence of 2-hydroxyethylhydrazine, an inhibitor of N-methyltransferase (17); iii) by pulse-chase experiments with CDP-ethanolamine (Fig. 1) showing that PE was the direct precursor; iv) by its stability to alkaline methanolysis, which hydrolyzed all the other phospholipids except the alklylether-glycerophospholipids (however we have demonstrated that, in our experiments, PE was not a donor of phosphoethanolamine during alkylether-PE syn-

thesis); v) by the possibility of methylation by S-adenosylmethionine, leading to sphingomyelin; we have demonstrated that this was essentially a direct methylation and that the succession of the reactions ceramide-phosphoethanolamine → PE → PC → sphingomyelin was very slight or nonexistent.

Addition of exogenous ceramide did not improve the rate of synthesis with DPPC (Table 1) or that with DOPE (Table 2). These results are in agreement with those of Voelker and Kennedy (4) who showed that, when PC was exogenous, sphingomyelin synthesis was independent of exogenous ceramide. However, we did not determine the roles of serum albumin and $MnCl_2$, essential to the enzymatic system studied by Marggraf et al. (3). Ceramide-phosphoethanolamine synthesis was dependent upon the amount of incubated membrane fraction and, consequently, on its content of PE:ceramide-phosphoethanolamine transferase (Table 3), and was limited by the amount of endogenous ceramide.

It may be concluded that there are two sites of sphingomyelin biosynthesis in rat hepatocytes and brain cells, the endoplasmic reticulum and plasma membrane, instead of either one or the other, as suggested by previous studies.

Ceramide-phosphoethanolamine formation implied its ability to be methylated into sphingomyelin. Table 4 shows that different membrane fractions were able to methylate ceramide-phosphoethanolamine, although N-methyltransferases were less active in brain membranes than in liver membranes. We have previously stated that this methylation occurred directly and that the reverse reactions, from ceramide-phosphoethanolamine to sphingomyelin, via PE and PC, were of little or no consequence.

In contrast to the majority of PL that were synthesized in endoplasmic reticulum, the highest activity of sphingomyelin synthesis was found in brain synaptic membrane. However, it may be noted that plasma membrane contained 17.7% of sphingomyelin compared to 3.4% for microsomes in hepatocytes (18), or 10% compared to 5.5% in brain cells from our experiments. Of course, sphingomyelin was not necessarily synthesized in situ; moreover, a nonspecific phospholipid exchange protein, found in hepatocytes (19), was able to transfer sphingomyelin.

Thus three important questions must be considered. 1. Is there a single transferase that accepts either PC or PE as a substrate, or are there two separate enzymes? 2. Are plasma membrane transferases identical to those of microsomes? A positive answer might explain their presence in the Golgi apparatus (3, 16). 3. What is the "sidedness" of sphingomyelin-synthesizing enzymes in each of the membranes that were studied? We hope that our research may soon answer these questions.

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