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Cellular and Enzymic Synthesis of Sphingomyelin†

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ABSTRACT: The synthesis of sphingomyelin was studied in baby hamster kidney cells and in subcellular fractions derived from rat liver. During pulse-chase experiments with [^{3}H]choline in tissue culture cells, the specific radioactivity of sphingomyelin continued to increase after the specific activities of phosphocholine and cytidine 5′-diphosphate choline (CDP-choline) had declined by a factor of 10. The addition of [^{3}H]methionine to cells that were grown in 1 mM dimethylethanolamine efficiently radiolabeled phosphatidylcholine (by methylation of phosphatidyldimethylethanolamine) and sphingomyelin but not phosphocholine or CDP-choline. Thus, the proximal donor of the phosphocholine moiety of sphingomyelin was not CDP-choline but probably phosphatidylcholine. These in vivo results prompted investigation of the enzymic synthesis using phosphatidyl[^{3}H]choline or [^{3}H]ceramide as substrates. With both substrates the subcellular fraction with the highest specific enzyme activity was the plasma membrane. When phosphatidyl[^{3}H]choline was used as the substrate, phospholipid exchange proteins were included in the reaction to effect the transfer of the labeled phospholipid from liposomes into the membrane bilayer in which the enzyme resided. Under these conditions the synthesis of sphingomyelin was almost completely dependent upon the addition of phospholipid exchange proteins. When [^{3}H]ceramide was used as the substrate, the addition of detergents was necessary for sphingomyelin synthesis. The use of phospholipid exchange proteins to introduce lipid substrates to membrane-bound enzymes may have much broader applicability.

An important problem concerning the synthesis of sphingomyelin has been the identification of the proximal donor of the phosphocholine moiety of this lipid. The initial studies of sphingomyelin synthesis in cell-free systems (Sribney & Kennedy, 1958) suggested that the final step of the synthetic scheme was the transfer of phosphocholine from cytidine 5′-diphosphate choline (CDP-choline) to ceramide. The physiological relevance of this observation was difficult to evaluate because the ceramide species most active as substrate possessed the threo configuration, while ceramides and sphingolipids isolated from tissues possessed the erythro configuration (Carter et al., 1956). Although Fujino et al. (1968) reported assay conditions for the synthesis of *erythro*-sphingomyelin from CDP-choline, other investigators (Ullman & Radin, 1974; van Golde et al., 1974) have been unable to reproduce these results. A study by Diringer et al. (1972) with SV-40 transformed cells suggested that the source of the phosphocholine moiety of sphingomyelin was phosphatidylcholine. However, in these experiments neither CDP-choline nor phosphocholine levels were measured. Ullman & Radin (1974) provided enzymic evidence that the phosphocholine moiety of sphingomyelin could be derived from phosphatidylcholine, but others have reported an inability to duplicate this work (Stoffel & Melzner, 1980). In the present study both cellular and enzymic approaches were used to identify the source of the phosphocholine moiety of sphingomyelin. Precursor-product analyses of tissue culture cells labeled with [^{3}H]choline and [^{3}H]methionine provided strong evidence that CDP-choline was not the immediate source of the phosphocholine moiety of sphingomyelin. The results further implicated phosphatidylcholine as the donor in vivo. The in vivo results were coupled to measurements of enzyme activities in well-defined subcellular fractions from rat liver. The enzyme phosphatidylcholine:ceramide phosphocholinetransferase was localized in the plasma membrane.

Materials and Methods

Tissue Culture. Baby hamster kidney (BHK 21) cells (ATCC CCL10) were grown in Dulbecco's modified Eagle's medium supplemented with glutamine (2 mM), penicillin (100

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units/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum (MA Bioproducts). The same media low in methionine (3 mg/L) or free of choline were made in the laboratory from amino acids, vitamins, and salts. For pulse–chase experiments, cells were plated at a density of 10^6/60-mm dish, 16–18 h prior to the addition of radiolabeled compound. In experiments with dimethylethanolamine, the cells were incubated in media supplemented with 1 mM base for 18 h prior to the labeling period. Cells were harvested in either phosphate-buffered saline or distilled water, with a rubber policeman.

Thin-Layer Chromatography. The solvent systems used for thin-layer chromatography were (A) chloroform/methanol/acetic acid/water (50:25:8:2.5 v/v), (B) chloroform/methanol/ammonia (65:35:8 v/v), (C) chloroform/methanol/acetic acid (90:2:8 v/v), and (D) methanol/0.85 M sodium chloride/ammonia (50:50:0.5 v/v).

Preparation of Radiolabeled Substrates. Phosphatidyl-[^{3}H]choline was prepared by incubating BHK 21 cells for 5 h in choline-free media supplemented with [*methyl*-^{3}H]choline (New England Nuclear) (1.5 μM, 80 Ci/mmol). The amount of phosphatidylcholine was estimated from replicate dishes to which no radiolabeled choline was added. The incoporation of radiolabel was usually 50%, and the specific activity of the isolated phosphatidylcholine ranged from 200 to 400 μCi/μmol. The total lipid extract (Bligh & Dyer, 1959) from the cells was treated with 0.5 unit of specific sphingomyelinase from *Staphylococcus aureus* (the generous gift of Dr. Ben Roelofsen, University of Utrecht, The Netherlands) (Zwaal et al., 1975) to remove labeled sphingomyelin. The radiolabeled phosphatidylcholine was further purified by preparative thin-layer chromatography on silica gel H plates (Applied Sciences) developed in solvent system A. Small unilamellar liposomes were prepared by sonicating the labeled phosphatidylcholine for 15 min, in 1-min bursts, followed by cooling. During sonication the liposome-containing vessel was maintained in an ice water bath and under a nitrogen atmosphere. Multilamellar liposomes were removed by centrifugation at 100000*g* for 1 h.

Radiolabeled ceramide (*N*-[9,10-^{3}H]palmitoylsphingosine) was prepared by acylating sphingosylphosphocholine and then removing the phosphocholine moiety. The [9,10-^{3}H]palmitic acid (16.8 Ci/mmol, New England Nuclear) was converted to the acyl chloride by refluxing at 50 °C in oxalyl chloride under nitrogen. The excess oxalyl chloride was removed by vacuum evaporation at 5 °C. Sphingosylphosphocholine was made according to the method of Kaller (1961) and purified to yield one spot after thin-layer chromatography with double development in solvent system B. Acylation of sphingosylphosphocholine was conducted at 37 °C for 24 h in freshly distilled chloroform/pyridine (9:1 v/v) (Chakrabarti & Khorana, 1975). The yield of [^{3}H]sphingomyelin was 40% of the theoretical value. The labeled sphingomyelin was treated with 0.3 N NaOH/methanol at 37 °C for 1 h, and the chloroform-extractable products were recovered. The [^{3}H]sphingomyelin was purified by preparative thin-layer chromatography using double development in solvent system A. The product was then treated with sphingomyelinase for 2 h by using the same conditions that were employed for the treatment of radiolabeled phosphatidylcholine. The resultant ceramide was then eluted from a 1-mL silicic acid column with chloroform/methanol (8:2). The purified [9,10-^{3}H]palmitoylsphingosine gave a single sharp peak of radioactivity when analyzed by thin-layer chromatography in solvent system C. Radiolabeled ceramide was also prepared by acylating *erythro-trans*-sphingosine (made from beef heart sphingomyelin) with the same conditions as used for sphingosylphosphocholine. The product was further purified by alkaline methanolysis and preparative thin-layer chromatography.

Product Analysis. Sphingomyelin was identified as the product of in vivo and in vitro reactions by alkaline methanolysis in 0.3 N NaOH/methanol at 37 °C for 1 h, followed by thin-layer chromatography in both acidic (system A) and basic (system B) solvent systems. Iodine staining was used to visualize compounds. Another method used to identify sphingomyelin was acid degradation according to the method of Kaller (1961). Virtually all of the sphingomyelin was recovered as sphingosylphosphocholine. The product of acid degradation was identified as sphingosylphosphocholine on the basis of its ninhydrin-positive character coupled with its R_f in thin-layer system A or B after double development.

Determination of Phosphocholine and CDP-choline. Total lipids were extracted from BHK 21 cells by the procedure of Bligh & Dyer (1959), and the upper phase was used to determine the amount of phosphocholine and CDP-choline. The recovery of these compounds was determined by adding a known amount of ^{14}C-labeled standards to the cells prior to harvesting. The upper phase from the extraction was lyophilized and the resultant residue dissolved in 1 mL of distilled water. The water-soluble compounds were chromatographed on a 1-mL column of Dowex AG 1X2 in the formate form. The column was washed with 3 mL of distilled water to elute choline and betaine. The adsorbed phosphocholine and CDP-choline were eluted with 3 mL of 0.04 N formic acid. At this stage the recovery of both compounds was nearly 100%. The formic acid eluates from the column were lyophilized, and the resultant residue was resuspended in 50 μL of methanol/0.085 M NaCl/ammonia (50:50:0.5 v/v). A 25-μL sample was banded on thin-layer plates (silica gel G, Brinkmann) and chromatographed in solvent system D. The 2 cm wide lanes of the thin-layer plates were cut into 5-mm strips, and the distribution of radiolabeled material was determined. Because the chemical amounts of phosphocholine and CDP-choline were small, the activity was expressed as a function of the amount of protein per dish. It was assumed that within a given experiment, the ratio of the chemical amounts of these compounds to the amount of protein did not change significantly.

Isolation of Subcellular Membranes. Plasma membrane fractions were isolated according to the procedure of Kervina & Fleischer (1974) or by a modification of the procedures of Neville (1968) and Berman et al. (1969) as reported by Lee et al. (1973). Mitochondria were isolated from homogenates as the fraction sedimenting between 2000*g* (10 min) and 12500*g* (10 min). Microsomes were isolated by centrifuging the postmitochondrial supernatant at 100000*g* for 1 h. Both mitochondria and microsomes were washed 3 times by recentrifugation. Golgi membranes were isolated by the single-step gradient procedures of Fleischer (1974).

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Inorganic phosphate was determined by the method of Bartlett (1959). The method of Song & Bodansky (1967) was used to measure 5′-nucleotidase activity. Glucose-6-phosphatase was measured by the method of Zakim & Vessey (1973) and indicated microsomal contamination (on the basis of enzyme specific activity) of plasma membrane preparations to be 10–15%. Acid phosphatase activity (Trouet, 1974) of plasma membrane fractions was negligible. All plasma membrane fractions used showed a minimum of 15-fold enrichment of the specific activity of 5′-nucleotidase, relative to the homogenate.

Partial Purification of Phosphatidylcholine Exchange Protein. The method chosen was drawn from several published procedures (DiCorleto et al., 1979; Poorthuis et al., 1980; Lumb et al., 1976). Briefly, the methods included preparation of a pH 5.1 supernatant fraction from rat liver homogenates, dialysis, chromatography on DE-52 (Whatman), and concentration by ammonium sulfate precipitation. Purified phosphatidylcholine exchange protein from beef liver was the generous gift of Dr. Donald B. Zilversmit, Cornell University. Exchange protein activity was determined essentially by the procedure of DiCorleto et al. (1979).

Assay System 1. The design of assay system 1 was to measure the transfer of phospho[^{3}H]choline from phosphatidyl[^{3}H]choline to ceramide. Phosphatidyl[^{3}H]choline was incoporated into subcellular membranes by the action of phosphatidylcholine exchange protein. Reactions were conducted in a final volume of 250 μL and contained 10 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] of pH 7.2, 50 mM NaCl, 0.5 μCi of phosphatidylcholine vesicles, 50–100 μg of membrane-fraction protein, and saturating amounts of purified or partially purified phosphatidylcholine exchange protein. After incubation at 37 °C for 60–90 min, the reactions were terminated by the addition of 1.5 mL of methanol/chloroform (2:1 v/v). After extraction (Bligh & Dyer, 1959) the lipids were dried and resuspended in 1 mL of 6 N HCl/butanol (1:1 v/v) and heated at 100 °C for 1 h. The solution was cooled and 0.5 mL of 3.5 N ammonia was added, followed by 0.5 mL of butanol. The resultant lower phase was removed and discarded, and the upper phase was washed twice with 1 mL of 0.1 N HCl saturated with butanol. A portion of the upper phase was counted in a scintillation counter.

Assay System 2. In assay system 2 transfer of phosphocholine from endogenous phosphatidylcholine to [^{3}H]ceramide was measured. The [^{3}H]sphingomyelin product was measured by a filter disk method. The reaction in a volume of 250 μL contained 10 mM Hepes buffer of pH 7.2, 50 mM NaCl, 0.125–0.5 μCi of *N*-[9,10-^{3}H]palmitoylceramide, and membranes (50–100 μg of protein). Radiolabeled ceramide was suspended in sodium taurodeoxycholate (4 mg/mL), and 5 μL was added to initiate the reaction. The lipid extract from the reaction was resuspended in 50 μL of chloroform, and 25 μL was applied to a 1.5 × 1 cm piece of silicic acid impregnated paper (Whatman SG-81). The solvent was allowed to evaporate, and the paper was transferred to a test tube. The paper was washed with 2 mL of chloroform/methanol (93:7) with gentle agitation for 4 min. At the end of the wash period the solvent was removed with a Pasteur pipet attached to a vacuum aspirator. A total of four washes was performed, after which the tubes were flushed with nitrogen to remove solvents. The papers were then transferred to scintillation vials. Addition of 0.5 mL of methanol to the vials prior to the addition of liquid scintillation fluid facilitated the elution of sphingomyelin product from the paper.

Measurement of Membrane Ceramide. The procedure of Siakotos et al. (1971) was modified to measure ceramide content of plasma membrane fractions. Ceramide was preparatively isolated by thin-layer chromatography and subjected to alkaline degradation. The resultant sphingosine was derivatized with trinitrobenzenesulfonic acid and its concentration determined by measuring absorbance at 410 nm.

Results

[^{3}H]Choline Labeling. The precursor–product relationship of choline-containing compounds was examined in pulse–chase experiments (Figure 1). During the 2-h labeling period,

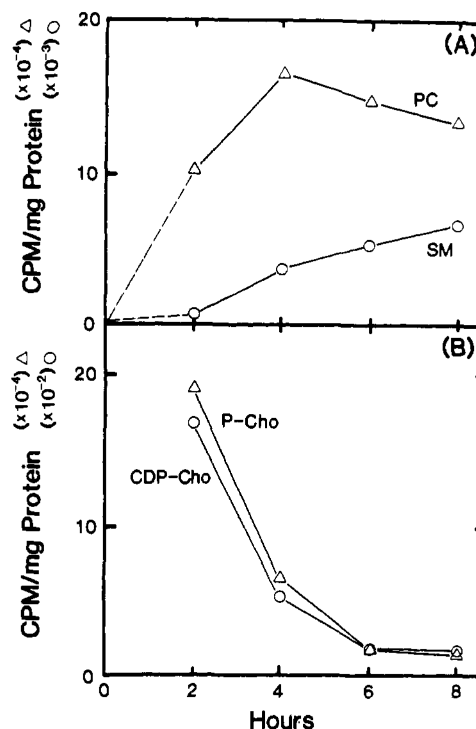

FIGURE 1: Pulse–chase labeling of BHK 21 cells with [*methyl*-^{3}H]choline. Cells were labeled for 2 h with [*methyl*-^{3}H]choline (1 μCi/mL, 28 μM), washed, and incubated in complete medium. (Panel A) Labeling pattern of phosphatidylcholine (PC) and sphingomyelin (SM). (Panel B) Labeling pattern of CDP-choline (CDP-Cho) and phosphocholine (P-Cho).

[^{3}H]choline was readily incorporated into phosphocholine, CDP-choline, phosphatidylcholine, and sphingomyelin. The initial rate of incoporation of radiolabel into sphingomyelin was about 1% of that found for phosphatidylcholine. The chase period was characterized by a rapid decline of the specific activity of both phosphocholine and CDP-choline. The specific activity of phosphatidylcholine increased at a reduced rate during the first 2 h of the chase and then declined. In contrast, the incorporation of [^{3}H]choline into sphingomyelin proceeded at nearly a linear rate for at least 6 h into the chase. These results implied that the synthesis of sphingomyelin was not directly dependent upon the synthesis of phosphocholine and CDP-choline.

[^{3}H]Methionine Labeling. For expansion upon the results obtained with [^{3}H]choline, the cells were labeled with [^{3}H]-methionine under conditions that allowed for clear differentiation between CDP-choline and phosphatidylcholine as the donor of the phosphocholine moiety of sphingomyelin. If phosphatidylcholine were the proximal donor of the phosphocholine moiety of sphingomyelin, then it should be possible to label sphingomyelin via a pathway that labels phosphatidylcholine without significantly labeling phosphocholine or CDP-choline. The sequential methylation of phosphatidylethanolamine to form phosphatidylcholine (Bremer & Greenberg, 1961) provided a metabolic route for performing such an experiment. The BHK 21 cells were incubated for 18 h in complete media supplemented with 1 mM dimethylethanolamine. These cells, which accumulated significant amounts of phosphatidyldimethylethanolamine, were washed and reincubated in media low in methionine, supplemented with [*methyl*-^{3}H]methionine of high specific radioactivity for 2 h, and then incubated in complete media. The [^{3}H]-methionine rapidly labeled phosphatidylcholine (Figure 2) while the labeling of phosphocholine and CDP-choline was very low. The labeling of sphingomyelin proceeded at a nearly

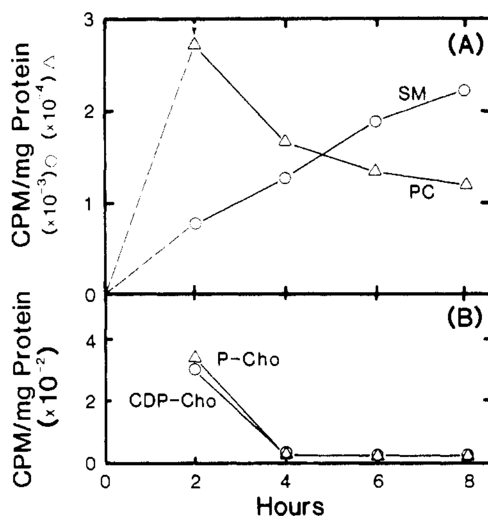

FIGURE 2: Pulse-chase labeling of BHK 21 cells with [*methyl*-^{3}H]methionine. Cells were incubated for 18 h in medium supplemented with 1 mM dimethylethanolamine and then labeled for 2 h with [*methyl*-^{3}H]methionine (10 μCi/mL, 20 μM). After the labeling period, the cells were washed and incubated in complete media. Abbreviations are the same as for Figure 1.

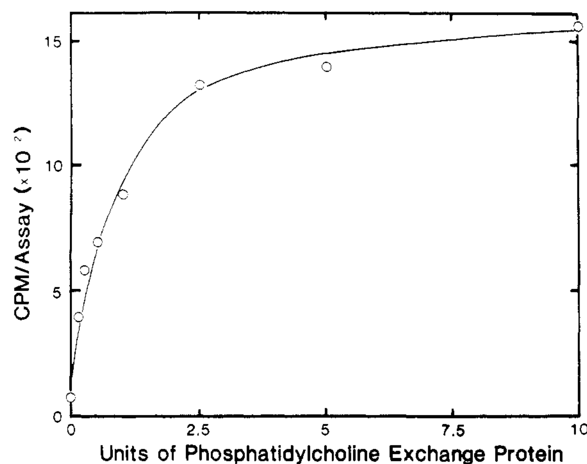

FIGURE 3: Dependence of sphingomyelin synthesis upon phospholipid exchange protein. Each reaction contained 50 μg of plasma membrane protein, 10 mM Hepes of pH 7.2, 50 mM NaCl, 0.5 μCi of phosphatidyl[^{3}H]choline vesicles, and varying amounts of purified beef liver phosphatidylcholine exchange protein. One unit is the transfer of 58 nmol of phosphatidylcholine/h. Reactions were at 37 °C for 1 h.

linear rate over the course of the experiment. This result strongly supports the conclusion that the choline moiety of sphingomyelin was not derived from CDP-choline but most probably from phosphatidylcholine.

Sphingomyelin Synthesis by a Plasma Membrane Phosphocholine Transferase. The results obtained from precursor-product studies with BHK 21 cells prompted the search for an enzyme that would synthesize sphingomyelin from phosphatidylcholine and ceramide. The putative role of phospholipid exchange proteins in the intracellular movement of phospholipids made them logical candidates for incorporating radiolabeled phosphatidylcholine into subcellular membranes for the purpose of studying sphingomyelin synthesis. In early experiments, when phosphatidyl[^{3}H]choline was the radiolabeled substrate, the synthesis of sphingomyelin exhibited almost complete dependence upon the pH 5.1 supernatant used as a crude source of phospholipid exchange proteins. For routine assays both pH 5.1 supernatant and partially purified phosphatidylcholine exchange protein supported the reaction adequately. So that the involvement of any other soluble proteins in the reaction could be ruled out, purified phosphatidylcholine exchange protein from beef liver was obtained from Donald B. Zilversmit, Cornell University. The dependence of the reaction upon the exchange protein is shown in Figure 3. Additional experiments were performed to demonstrate that the phosphatidyl[^{3}H]choline had actually been transferred from the nonsedimentable (100000*g* for 60 min) unilamellar vesicles to the sedimentable (12000*g* for 10 min) plasma membrane fraction. When saturating amounts of phosphatidylcholine exchange protein were used, sphingomyelin synthesis was linear for 90 min. Product formation was also linear with plasma membrane protein concentrations between 20 and 200 μg/incubation.

From preliminary experiments, it also became obvious that the highest enzyme activity was to be found in the plasma membrane fraction. The reaction conditions were optimized in the plasma membrane fraction and then measured more carefully in other membrane fractions. The distribution of sphingomyelin synthase activity among the various subcellular membrane fractions is shown in Table I. The specific activity units are expressed in terms of radioactivity per milligram of protein because the endogenous phosphatidylcholine and

Table I: Subcellular Distribution of Phosphocholine Transferase[a]

	sp act. (arbitrary units/mg of protein)		
	phosphocholine transferase		
fraction	assay system 1[c]	assay system 2[d]	5'-nucleotidase[b]
homogenate (4)	0.67 ± 0.33	5.5 ± 0.92	0.048 ± 0.005
mitochondria (6)	3.86 ± 1.22	8.9 ± 0.70	0.140 ± 0.021
microsomes (6)	1.20 ± 0.39	6.6 ± 0.70	0.090 ± 0.003
Golgi (2)	4.80	9.1	ND
plasma membrane (6)	36.04 ± 1.4	42.9 ± 3.96	0.760 ± 0.063

[a] Numbers in parentheses indicate the number of independent determinations. Maximum sphingomyelin synthesis in assay system 1 is 3.5 nmol h^{-1} (mg of protein)$^{-1}$. Maximum sphingomyelin synthesis in assay system 2 is 2.75 nmol h^{-1} (mg of protein)$^{-1}$. ND, not determined. [b] 1 unit = 1 μmol/min. [c] 1 unit = 10^3 cpm/h. [d] 1 unit = 10^4 cpm/h.

ceramide pools were not routinely measured in all fractions. In the plasma membrane fraction the average phosphatidylcholine content was 320 nmol/mg of protein, and the average ceramide concentration was 28.7 nmol/mg of protein. It was assumed that the phosphatidyl[^{3}H]choline transferred to the plasma membrane mixed ideally with the total pool of phosphatidylcholine. When [^{3}H]ceramide was used as the radiolabeled substrate, it was also assumed that ideal mixing occurred with the total membrane pool. On the basis of these assumptions the rate of sphingomyelin synthesis was determined to be 3.5 nmol h^{-1} (mg of protein)$^{-1}$ when phosphatidyl[^{3}H]choline was the substrate and 2.75 nmol h^{-1} (mg of protein)$^{-1}$ when [^{3}H]ceramide was the substrate.

Identification of the Products of Enzymic Synthesis. The product of the transferase reaction was identified as sphingomyelin on the basis of its (a) stability to alkali, (b) migration with authentic sphingomyelin standards on thin-layer chromatograms in both acidic and basic solvent systems, (c) degradation by a specific sphingomyelinase from *S. aureus*, and (d) conversion to the ninhydrin-positive product sphingosylphosphocholine following acid degradation. The thin-layer chromatography profile of radiolabeled products of the enzyme reactions is shown in Figure 4.

pH and Salt Dependence. The pH optimum occurred between 7.0 and 7.5. The reaction rate increased with increasing

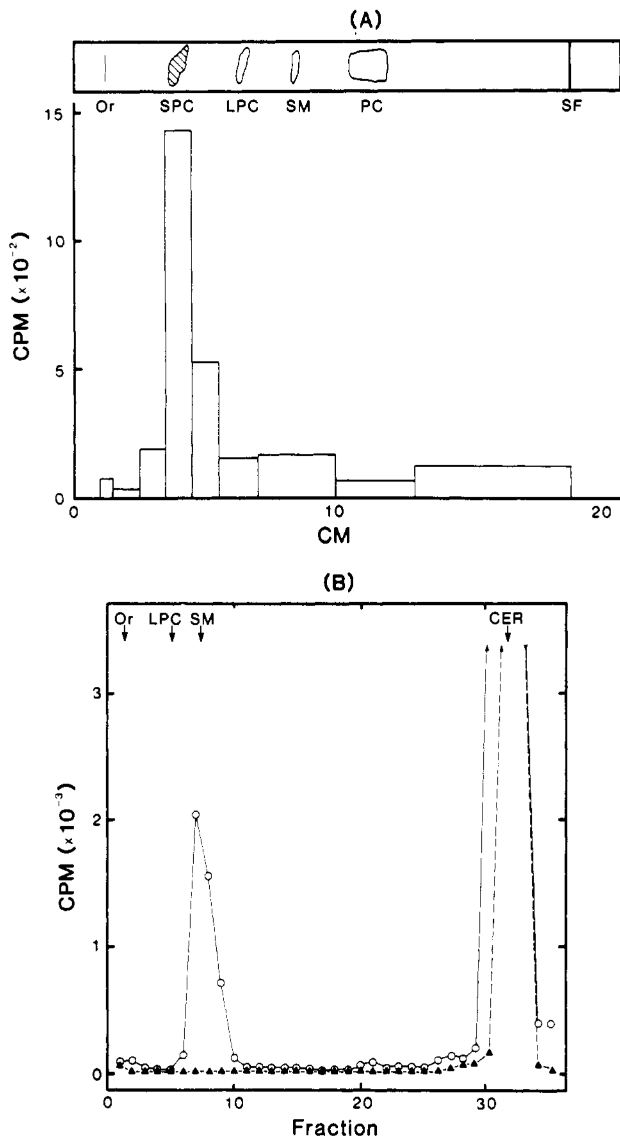

FIGURE 4: Identification of sphingomyelin as the product of enzymic synthesis. (Panel A) The alkali-stable product from assay system 1 that cochromatographed with sphingomyelin in solvent system A was treated with 6 N HCl/butanol (1:1) for 1 h at 100 °C and then chromatographed by double development in solvent system B. Or, origin; SPC, sphingosylphosphocholine; LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine. The shading indicates staining with ninhydrin. Recovery of radiolabel was better than 90%. (Panel B) Thin-layer chromatogram of alkali-stable lipids from assay system 2. Abbreviations are the same as in panel A; Cer, ceramide. (O) Standard reaction products; (▲) products recovered from pooled enzyme blank reactions. The chromatogram was developed twice in solvent system A. Recovery of radiolabel was better than 95%.

salt concentration and was 3-fold higher in the presence of 50 mM NaCl or KCl than the rate obtained without added salt. Since the reaction was also dependent upon phospholipid exchange protein, the effects of salt concentration and pH upon phospholipid exchange were also examined. Phospholipid exchange activity was unaffected by the variations of pH and salt concentration that affected sphingomyelin synthesis. The enzyme exhibited no requirement for the added divalent cations Ca, Mg, Mn, or Zn, and the reaction rate was unaffected by EDTA (ethylenediaminetetraacetic acid) in concentrations up to 5 mM.

Effect of Possible Intermediates. The possibility that phosphocholine or choline might exist as exchangeable intermediates in the reaction was considered. Inclusion of phosphocholine, choline, or CDP-choline as possible isotope traps, at concentrations of 1 mM, was without effect upon enzyme activity.

Transfer of [^{33}P]Phosphocholine. In addition to phosphatidyl[^{3}H]choline, [^{33}P]phosphatidylcholine was used as a substrate in the reaction. The rate of formation of [^{33}P]-sphingomyelin from [^{33}P]phosphatidylcholine [3 nmol h^{-1} (mg of protein)$^{-1}$] was comparable to that found for the ^{3}H-labeled substrate. Thus, the entire phosphocholine moiety was transferred to ceramide.

Sphingomyelin Synthesis from [^{3}H]Ceramide. When [^{3}H]ceramide was used as the radiolabeled substrate to measure sphingomyelin synthesis, the reaction required the addition of detergents. Levels of detergents that are normally used to solubilize membrane enzymes inhibited the reaction. The best results were obtained if the [^{3}H]ceramide was suspended in detergent solutions around the critical micellar concentration (Helenius et al., 1979) and then diluted 50-fold into the reaction. Enzyme activity expressed as a function of the final detergent concentration is shown in Figure 5. Unlike the assay with [^{3}H]phosphatidylcholine, the assay with [^{3}H]ceramide was not linear with time between 15 and 90 min. For accurate measurement of initial rates, the 15-min time interval should be used.

Ceramide Dependence of Sphingomyelin Synthesis. When [^{3}H]ceramide was used to follow the reaction, the initial rate was dependent upon the exogenous ceramide concentration (Figure 6). The results presented in Figure 6 are an underestimate of the actual rates (especially at lower concentrations of added ceramide) since the abscissa values do not include the concentration of endogenous ceramide, which would be expected to dilute the specific radioactivity of [^{3}H]ceramide. In contrast to these observations, synthesis of sphingomyelin measured with phosphatidyl[^{3}H]choline appeared to be independent of added ceramide. Addition of ceramide in various forms (ethanol dilution, detergent dilution, or as sonicated suspensions with and without phospholipid) did not increase the rate of sphingomyelin synthesis from phosphatidyl[^{3}H]choline.

Discussion

The results presented in Figures 1 and 2 provided strong evidence that, in vivo, the proximal donor of the phosphocholine moiety of sphingomyelin was not CDP-choline but most probably phosphatidylcholine. This conclusion was based upon the data of Figure 1, where between 2 and 8 h of chase the specific activity of CDP-choline declined by a factor of 10, the specific activity of phosphatidylcholine reached a maximum and then declined by 20%, and the specific activity of sphingomyelin continued to increase at a near linear rate. Further support for this conclusion came from the results shown in Figure 2 in which the phosphocholine moiety of sphingomyelin was efficiently labeled as a consequence of the methylation of phosphatidyldimethylethanolamine. In addition, the recent work of Esko & Raetz (1980) with temperature-sensitive mutants of Chinese hamster ovary cells that were incapable of synthesizing normal levels of CDP-choline indicated that sphingomyelin synthesis was normal at the nonpermissive temperature. Collectively, these observations make it unlikely that CDP-choline functions as a substrate in the synthesis of sphingomyelin.

The ability of phosphatidyl[^{3}H]choline to function as a substrate for sphingomyelin synthesis in vitro was almost completely dependent upon the presence of phosphatidylcholine exchange protein. This observation suggests that the enzyme was incapable of catalyzing head-group transfer from phosphatidylcholine molecules that resided within separate bilayers.

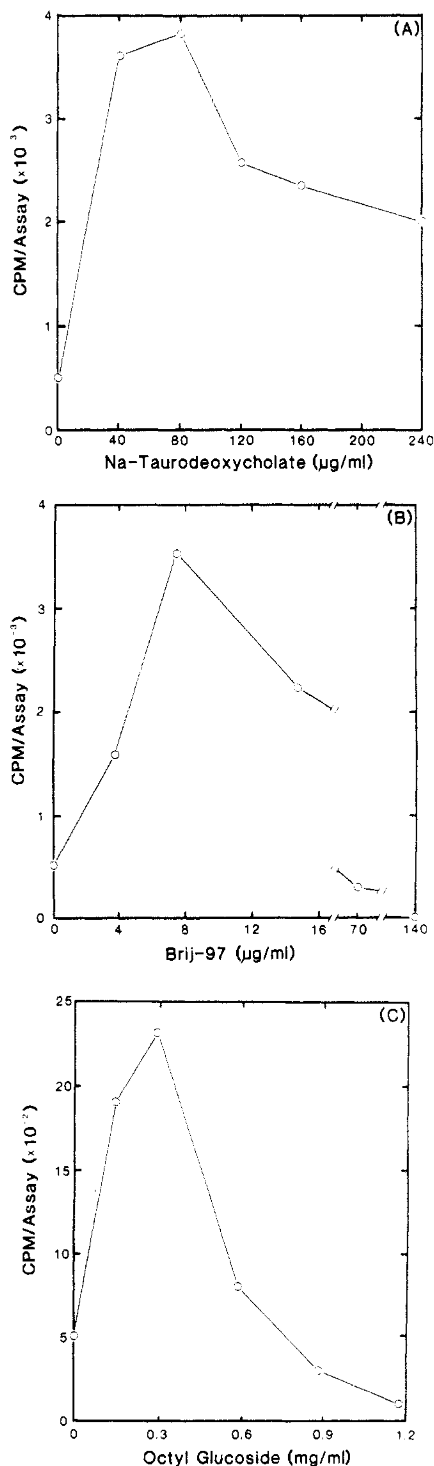

FIGURE 5: Detergent dependence of sphingomyelin synthesis from [^{3}H]ceramide. Each reaction mixture contained 50 μg of plasma membrane protein, 10 mM Hepes of pH 7.2, 50 mM NaCl, 0.5 μCi of [^{3}H]ceramide, and varying final concentrations of detergents. The detergents were mixed with [^{3}H]ceramide, and 5 μL was added to each assay tube. Incubations were at 37 °C for 60 min.

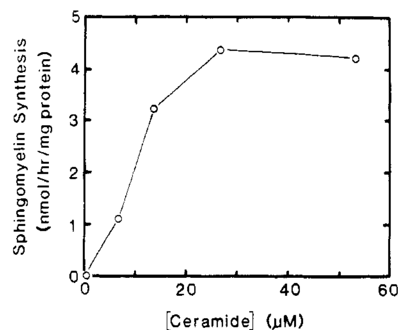

FIGURE 6: Dependence of sphingomyelin synthesis upon added ceramide. Each reaction mixture contained 50 μg of protein and was incubated at 37 °C for 15 min. Sodium taurodeoxycholate was used as the detergent at a final concentration of 80 μg/mL. The abscissa values do not include the concentration of endogenous ceramide.

The rationale for using phospholipid exchange proteins in the enzyme reaction was that they might also function in this manner in the synthesis of sphingomyelin in vivo. It is anticipated that phospholipid exchange proteins can be employed in a variety of other enzyme systems that utilize lipid substrates.

The plasma membrane was the subcellular fraction with the highest specific enzyme activity for sphingomyelin synthesis. This is in contrast to the majority of the other phospholipids that are synthesized in the endoplasmic reticulum. The localization of sphingomyelin synthesis to the plasma membrane coupled to the relative sluggishness of phospholipid exchange proteins with this lipid (Zilversmit & Hughes, 1977; Bloj & Zilversmit, 1977) may account for the enrichment of sphingomyelin in plasma membranes.

Marggraf et al. (1981) recently reported that plasma membrane fractions from SV-40 transformed mouse fibroblasts also exhibited sphingomyelin synthesizing activity. Their enzyme system differed from ours in that it required 20 mg/mL albumin and 3 mM $MnCl_2$ for activity and did not employ phospholipid exchange protein when phosphatidylcholine was the substrate. These authors also did not report any data for the conversion of radiolabeled ceramide into sphingomyelin. In the present study no divalent cation requirement was observed, and sphingomyelin synthesis from either [^{3}H]ceramide or phosphatidyl[^{3}H]choline was insensitive to the addition of EDTA. Our conclusions are similar to those drawn by Ullman & Radin (1974) but substantially expand upon their observations. We have defined the parameters for sphingomyelin synthesis in a more general system and identified the subcellular fraction responsible for the observed activity. Their use of animals in a highly specialized nutritional state (preweaned) may account for the fact that others (Stoffel & Melzner, 1980) have reported an inability to reproduce their results. The assay systems developed in this study are easy to manipulate and should provide a firm basis for purifying the enzyme and understanding its role in membrane biogenesis.

The ability of isolated plasma membrane preparations to synthesize sphingomyelin from phosphatidylcholine in the absence of added ceramide was surprising. Measurements of the endogenous ceramide indicated that it comprised 1–2% of the plasma membrane lipid. When phosphatidyl[^{3}H]choline was used to measure sphingomyelin synthesis, only 10% of the endogenous plasma membrane pool of ceramide was converted to product, and the addition of exogenous ceramide was without effect. However, when [^{3}H]ceramide was the substrate, the initial rate of the reaction was dependent upon the concentration of added ceramide. The reasons for the differences between the two substrates are presently under investigation. While the manuscript was in preparation, Bernert & Ullman (1981) reported the partial characterization of a microsomal enzyme catalyzing the synthesis of sphingomyelin.

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Calcium-Independent Stimulation of *Bordetella pertussis* Adenylate Cyclase by Calmodulin†

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ABSTRACT: *Bordetella pertussis* produces an extracellular adenylate cyclase activity that is present in the culture medium of exponentially growing cells. We have determined that calmodulin (CaM) stimulates this enzyme both in the presence and in the absence of free Ca^{2+}. In the presence of 90 μM Ca^{2+} half-maximal stimulation of the enzyme occurred at 95 pM calmodulin. Comparable levels of calmodulin stimulation were observed when free Ca^{2+} levels were minimized by using EGTA-containing buffers. However, the concentration of CaM required for half-maximal stimulation of *B. pertussis* adenylate cyclase in the presence of 1 nM free Ca^{2+} was 24 nM. The apparent affinity of the enzyme for calmodulin was also significantly enhanced by Mn^{2+}. In addition, troponin I inhibited calmodulin stimulation of the bacterial adenylate cyclase. Photoaffinity cross-linking experiments using azido[^{125}I]calmodulin and *B. pertussis* adenylate cyclase revealed only one major cross-linked product having a molecular weight of 97 000. It is proposed that the catalytic subunit of the calmodulin-sensitive adenylate cyclase is 77 000.

Bordetella pertussis is a small Gram-negative bacilli that is the pathogen responsible for whooping cough (Olson, 1975; Jawetz et al., 1978). The organism adheres to and multiplies on the surface of tracheal and bronchial epithelium, interfering with ciliary function (Linnemann, 1978). Although the molecular basis for *B. pertussis* pathology is undefined, culture media of growing *B. pertussis* contain a number of biologically active components (Morse, 1976). The culture medium of exponentially growing *B. pertussis* accumulates a soluble adenylate cyclase that is insensitive to α-keto acids and guanyl nucleotides (Hewlett et al., 1976; Hewlett & Wolff, 1976). It has been reported that this enzyme is monomeric with a molecular weight of 70 000 on $NaDodSO_4$[1] gels and sucrose

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