Sphingomyelin Synthesis in Rat Liver Occurs Predominantly at the cis and medial Cisternae of the Golgi Apparatus*

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The intracellular site of sphingomyelin (SM) synthesis was examined in subcellular fractions from rat liver using a radioactive ceramide analog N-[(1-14C)hexanoyl]-d-erythro-sphingosine. This lipid readily transferred from a complex with bovine serum albumin to plasma membranes without disrupting the membranes, and was metabolized to radioactive SM. To prevent degradation of the newly synthesized SM to ceramide, all experiments were performed in the presence of EDTA to minimize neutral sphingomyelinase activity and at neutral pH to minimize acid sphingomyelinase activity.

An intact Golgi apparatus fraction gave an 80-90-fold enrichment of SM synthesis and a 58-83-fold enrichment of galactosyltransferase activity. Controlled trypsin digestion demonstrated that SM synthesis was localized to the lumen of intact Golgi apparatus vesicles. Although small amounts of SM synthesis were detected in plasma membrane and rough microsome fractions, after accounting for contamination by Golgi apparatus membranes, their combined activity contributed less than 13% of the total SM synthesis in rat liver.

Subfractions of the Golgi apparatus were obtained and characterized by immunoblotting and biochemical assays using cis/medial (mannosidase II) and trans (sialyltransferase and galactosyltransferase) Golgi apparatus markers. The specific activity of SM synthesis was highest in enriched cis and medial fractions but far lower in a trans fraction. We conclude that SM synthesis in rat liver occurs predominantly in the cis and medial cisternae of the Golgi apparatus and not at the plasma membrane or endoplasmic reticulum as described previously.

Sphingomyelin (SM) is ubiquitous in animal tissues and has been found in almost every cell and membrane examined.

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Rats were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA) or from Taconic Farms, Inc. (Germantown, NY). Ultrapure sucrose was purchased from Schwarz/Mann. [3H]UDP-Gal (40.4 Ci/mmol) was from Du Pont-New England Nuclear. 125I-Labeled protein A was from Amersham Corp. Sulfotransferase-conjugated bovine serum albumin (SRhBSA) was from Molecular Probes Inc. (Eugene, OR) and trypsin (TRL-3 grade) was from Worthington. Antiserum directed against rat liver Golgi apparatus mannose-2,6-sialyltransferase (21) was a gift from Dr. K. Moremen (Massachusetts Institute of Technology), and affinity-purified rabbit IgG directed against rat liver a2,6-sialyltransferase (22) was a gift from Dr. G. Hart (Johns Hopkins School of Medicine). Solvent were from Burdick & Jackson Laboratories Inc. (Muskegon, MI), and, unless indicated, all other chemicals and reagents were from Sigma.

EXPERIMENTAL PROCEDURES

Materials

Rats were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA) or from Taconic Farms, Inc. (Germantown, NY). Ultrapure sucrose was purchased from Schwarz/Mann. [3H]UDP-Gal (40.4 Ci/mmol) was from Du Pont-New England Nuclear. 125I-Labeled protein A was from Amersham Corp. Sulfotransferase-conjugated bovine serum albumin (SRhBSA) was from Molecular Probes Inc. (Eugene, OR) and trypsin (TRL-3 grade) was from Worthington. Antiserum directed against rat liver Golgi apparatus mannose-2,6-sialyltransferase (21) was a gift from Dr. K. Moremen (Massachusetts Institute of Technology), and affinity-purified rabbit IgG directed against rat liver a2,6-sialyltransferase (22) was a gift from Dr. G. Hart (Johns Hopkins School of Medicine). Solvent were from Burdick & Jackson Laboratories Inc. (Muskegon, MI), and, unless indicated, all other chemicals and reagents were from Sigma.

Radioactive Lipids

The N-hydroxysuccinimide ester of 1-14C hexanoic acid (30.9 mCi/mmol) was obtained from Sigma or was prepared as described.
Preparation of BSA-Lipid Complexes

Defatted BSA-[14C]hexanoyl lipid complexes were prepared as described (25), except that 50 mM Tris (pH 7.4) was used; complexes were centrifuged in 0.25 M sucrose, and then rehomogenized in 0.25 M sucrose-hexanoyl lipid. After 20 min at room temperature, complexes were dialyzed against 50 mM Tris (pH 7.4) for 12-18 h at 4 °C. To minimize loss of material when small amounts of defatted BSA-[14C]hexanoyl SM were prepared, complexes were not dialyzed. Complexes were stored at -20 °C.

Subcellular Fractionation

Golgi Apparatus—An intact Golgi apparatus fraction was prepared (26) with some modifications. Male Sprague-Dawley rats (100-170 g) were starved for 18-24 h. After decapitation, livers were rapidly removed, perfused with ice-cold saline (0.9%, w/v), and homogenized with a loose-fitting Dounce homogenizer. The suspension was filtered through four layers of gauze to remove connective tissue and adjusted to 1.02 M sucrose, and 7 ml of 1.0 M sucrose by addition of 2.0 M sucrose to the homogenate. The homogenate was filtered through four layers of gauze to remove connective tissue and adjusted to 1.02 M sucrose with or without MgCl2 (5 mM), EDTA was included in the incubation buffer (TKE buffer) (see "Results"). Samples were warmed to 37 °C for 10 min before addition of defatted BSA-[14C]hexanoyl lipid. The reaction was stopped with 2-3 ml of chloroform/methanol (1/2, v/v) and the samples were immediately placed on ice. Lipids were extracted by the procedure of Bligh and Dyer (34).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (35) was performed using a resolving gel of 7.5% acrylamide. For detection of mannosidase II by immunoblot analysis, samples were heated at 65 °C for 5 min in sample buffer (8% glycerol, v/v; 1% SDS, w/v; 0.17 M Tris (pH 6.8)) containing 2-mercaptoethanol (5.5%, v/v); for sialyltransferase, samples were heated at 37 °C for 5 min in the absence of reducing agent. Immunoblotting was performed after wet electroblotting to nitrocellulose paper for 12-18 h at 100-200 mA (36). Nonspecific sites on the nitrocellulose were first blocked by BSA (1 mg/ml), incubated with anti-mannosidase II antibodies (1:4000 dilution) or anti-sialyltransferase antibody (1 μg/ml), washed (1% Tween 20, 150 mM NaCl, 0.05% Nonidet P-40), and incubated again for 1 hr at room temperature. Immunoblotting was performed after wet electroblotting to nitrocellulose paper for 12-18 h at 100-200 mA (36).

Permeabilization of Golgi Vesicles

The intact Golgi apparatus fraction was dialyzed overnight against deionized water. The integrity of the Golgi apparatus vesicles was determined by measuring the sensitivity of galactosyltransferase to trypsin (32).

Trypsin Treatment of Golgi Vesicles

An aliquot of the Golgi apparatus fraction was incubated with trypsin (ratio of Golgi apparatus protein to trypsin, 2/1, w/w) at 30 °C for 10 min (33) in the appropriate buffer. The reaction was stopped by addition of five times the amount of soybean trypsin inhibitor to trypsin, 2/1, w/w for 10 min at 30 °C. In control incubations only soybean trypsin inhibitor was added.

Incubation of Radioactive Lipids with Liver Fractions

SM synthesis and sphingomyelinase activity were assayed by incubating aliquots of liver fractions with either [14C]hexanoyl Cer or [14C]hexanoyl SM, and analyzing, respectively, the amounts of [14C]hexanoyl SM or [14C]hexanoyl Cer produced. No other lipid products were formed under the conditions used. Fractions were diluted in 25 mM KCl, 50 mM Tris (pH 7.4) (TKE buffer) to the desired protein concentration in a final volume of 500 μl or 1 ml. In most cases 0.5 mM EDTA was included in the incubation buffer (TKE buffer) (see "Results"). Samples were warmed to 37 °C for 10 min before addition of defatted BSA-[14C]hexanoyl lipid. The reaction was stopped with 2-3 ml of chloroform/methanol (1/2, v/v) and the samples were immediately placed on ice. Lipids were extracted by the procedure of Bligh and Dyer (34).

Analysis of Lipid Products

Lipid extracts were dried under a stream of nitrogen and then dried in vacuo and separated by analytical TLC on Silica Gel 60 plates (Merck) using chloroform/methanol/15 mM CaCl2 (60/35/8, v/v) as the developing solvent. [14C]Hexanoyl lipids were identified after autoradiography by comparison with authentic [14C]hexanoyl lipid standards. The RF values for [14C]hexanoyl Cer and [14C]hexanoyl SM were 0.75 and 0.25, respectively. [14C]Hexanoyl lipids were recovered from the plates by scraping and radioactivities were determined by liquid scintillation counting in a Packard Instrument Co. Minaxi (Tri-Carb 4000 series) scintillation counter using Beckman Ready Safe scintillant. Background radioactivity in the area corresponding to [14C]hexanoyl SM was measured by incubating [14C]hexanoyl Cer for 5 or 10 min at 37 °C in 500 μl of TKE buffer in the absence of any liver fractions, extracting, separating by TLC, and measuring, respectively, the amounts of [14C]hexanoyl SM in the corresponding experimental lane. This subtraction was particularly significant when small amounts (i.e., 10-100 pmol) of [14C]hexanoyl SM were synthesized. The efficiencies of extraction and counting were identical for both [14C]hexanoyl Cer and [14C]hexanoyl SM.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (35) was performed using a resolving gel of 7.5% acrylamide. For detection of mannosidase II by immunoblot analysis, samples were heated at 65 °C for 5 min in sample buffer (8% glycerol, v/v; 1% SDS, w/v; 0.17 M Tris (pH 6.8)) containing 2-mercaptoethanol (5.5%, v/v); for sialyltransferase, samples were heated at 37 °C for 5 min in the absence of reducing agent. Immunoblotting was performed after wet electroblotting to nitrocellulose paper for 12-18 h at 100-200 mA (36). Nonspecific sites on the nitrocellulose were first blocked by BSA (1 or 2%, w/v) in 150 mM NaCl, 0.05% Nonidet P-40, 10 mM Tris (pH 7.4) (2 hr, room temperature). Nitrocellulose blots were then incubated overnight with anti-mannosidase II antiserum (1:4000 dilution) or anti-sialyltransferase antibodies in PBS (0.1 M NaCl, 0.05% Tween 20, 0.05% Nonidet P-40, 10 mM Tris (pH 7.4)), incubated with 125I-labeled protein A, washed again, dried, detected by autoradiography, and quantified by scanning densitometry.
Miscellaneous Procedures

**Fluorescence Measurements**—Sulforhodamine fluorescence ($F_{\text{SRhBSA}}$ = 585; $F_{\text{SRhBSA-SRM}} = 610$) was quantified using a SLM Aminco 8000 spectrophotofluorimeter.

**Electron Microscopy**—Samples were fixed in 2% glutaraldehyde ($v/v$), 0.8% formaldehyde ($v/v$) in 0.1 M sodium cacodylate (pH 7.4) either before or after pleating. The pellet was postfixed in 1% OsO4, ($v/v$), dehydrated in a graded ethanol series, stained $en$ bloc with uranyl acetate (0.5%, $v/v$), and embedded in Epon 812/Quetol 651. Thin sections were prepared and stained using lead citrate prior to examination in a JEOI 100 s electron microscope.

**Miscellaneous Assays**—Protein concentrations were measured (37) with BSA as a standard, using dye reagent from Bio-Rad. Galactosyltransferase was assayed (38) using trypsin inhibitor type III-O (Ovomucoid, Sigma) as the acceptor. Alkaline phosphodiesterase (39), glucose-6-phosphatase (40), and lipid concentrations determined by phosphorous analysis (41) were measured as described.

**RESULTS**

**Insertion of $[^{14}C]$Hexanoyl Cer and $[^{14}C]$Hexanoyl SM into Rat Liver Membranes**—To establish that the radioactive sphingolipid analogs used in this study spontaneously transferred between BSA-lipid complexes and subcellular fractions from rat liver, SRhBSA-$[^{14}C]$hexanoyl Cer or SRhBSA-$[^{14}C]$hexanoyl SM were incubated with an intact Golgi apparatus fraction (see below), a liver homogenate, or an enriched PM fraction (see below). Transfer of $[^{14}C]$hexanoyl lipid was determined by measuring $^{14}C$ radioactivity and sulforhodamine fluorescence in the pellet obtained after centrifugation. Most of the $[^{14}C]$hexanoyl Cer and $[^{14}C]$hexanoyl SM, but only a small amount of SRhBSA, was associated with the pellet obtained from Golgi apparatus membranes (Table I) or with the pellet obtained from the liver homogenate and the enriched PM fraction (not shown). The small amount of SRhBSA (5–6%) associated with the pellet was probably an overestimation of the amount of adsorbed SRhBSA as the pellet was not washed prior to fluorescence measurements. These results indicate that the $[^{14}C]$hexanoyl sphingolipids spontaneously transferred to membranes and that transfer of the $[^{14}C]$hexanoyl sphingolipid was not due to adsorption of the SRhBSA-$[^{14}C]$hexanoyl sphingolipid complex to membranes. Since the amount of $[^{14}C]$hexanoyl sphingolipids transferred was similar for all three fractions, we assume that the synthesis of $[^{14}C]$hexanoyl sphingolipids described below was not limited by the transfer of substrate from BSA to membranes.

To determine if incorporation of $[^{14}C]$hexanoyl Cer disrupted the membranes of the Golgi apparatus, the trypsin sensitivity of galactosyltransferase, a luminal Golgi enzyme (32), was examined. Addition of up to 5 nmol of $[^{14}C]$hexanoyl Cer to Golgi membranes (25 μg of protein) had no significant effect on the sensitivity of galactosyltransferase to trypsin compared to control samples, demonstrating that Golgi vesicles remained intact upon addition of $[^{14}C]$hexanoyl Cer. In contrast, the addition of Triton X-100 (0.5% final concentration) rendered 98% of the galactosyltransferase activity sensitive to trypsin.

**Effects of EDTA and Mg$^{2+}$ on $[^{14}C]$Hexanoyl Sphingolipid Metabolism**—Conditions were established in which degradation of $[^{14}C]$hexanoyl SM to $[^{14}C]$hexanoyl Cer by endogenous sphingomyelinases (18–20) was minimized in liver fractions, permitting accurate quantification of the synthesis of $[^{14}C]$hexanoyl SM described below.

| Radioactive SRhBSA-lipid complex | % Radioactivity in pellet | % SRh fluorescence in pellet |
|----------------------------------|-------------------------|----------------------------|
| SRhBSA-$[^{14}C]$hexanoyl Cer     | 82.1 ± 1.6              | 5.9 ± 0.2                  |
| SRhBSA-$[^{14}C]$hexanoyl SM     | 94.8 ± 0.8              | 6.6 ± 0.4                  |

**Fig. 1.** Effects of Mg$^{2+}$ and EDTA on $[^{14}C]$hexanoyl SM synthesis and on sphingomyelinase activity in a liver homogenate. A rat liver homogenate was prepared in 0.25 M sucrose, 25 mM KCl, 50 mM Tris (pH 7.4). 500 or 300 μg of protein of the homogenate were incubated with 1.0 nmol of $[^{14}C]$hexanoyl Cer or 0.5 nmol of $[^{14}C]$hexanoyl SM, respectively, in the presence of MgCl$_2$ (A) or EDTA (B), in a final volume of 500 μl of TK buffer. After 10 min at 37 °C, the reaction was stopped and the lipids analyzed. SM synthesis was determined by measuring the amount of $[^{14}C]$hexanoyl SM formed (△) from $[^{14}C]$hexanoyl Cer. Sphingomyelinase activity was determined by measuring the amount of $[^{14}C]$hexanoyl Cer formed (○) from $[^{14}C]$hexanoyl SM. and expressed as a percentage of the original activity in the homogenate.

Sphingomyelin synthesis at the Golgi apparatus membranes

A Golgi apparatus fraction (100 μg of protein) was incubated with 1.0 nmol of SRhBSA-$[^{14}C]$hexanoyl Cer or 0.25 nmol of SRhBSA-$[^{14}C]$hexanoyl SM (ratio of SRhBSA to $[^{14}C]$hexanoyl lipid, 1:1), for 10 min at 37 °C in 1 ml of TK buffer. After centrifugation (Beckman TL-100 centrifuge, 263,000 × g, 10 min, 4 °C) $^{14}C$ radioactivity and SRh fluorescence were measured in the pellet and expressed as a percentage of that in the original SRhBSA-$[^{14}C]$hexanoyl lipid complex. Data represent the mean ± S.D. of three experiments.

This is also supported by the observation that the amount of $[^{14}C]$hexanoyl SM formed in an intact Golgi apparatus fraction was identical when $[^{14}C]$hexanoyl Cer was introduced from a complex with BSA, or from unilamellar liposomes generated from either dioleoylphosphatidylcholine or lipids extracted from the Golgi apparatus fraction (not shown).
Sphingomyelin Synthesis at the Golgi Apparatus

The organelle in which most of each enzyme activity is found is noted in parentheses. Results are expressed as the mean ± S.D. of four gradients. The absolute values in fraction b were: protein, 0.39 ± 0.07 mg/g liver; [3H]hexanoyl SM synthesis, 1.30 ± 0.1 nmol/min/mg protein; galactosyltransferase, 3.26 ± 0.8 nmol/min/mg protein; glucose-6-phosphatase, 102 ± 22 nmol/min/mg protein; alkaline phosphodiesterase, 308 ± 52 nmol/min/mg protein.

### Table II

| Fractions | Protein (mg/g liver) | Lipid phosphorous (nmol) | [3H]Hexanoyl SM synthesis* (nmol/min/mg protein) | Galactosyltransferase (Golgi) (nmol/min/mg protein) | Glucose-6-phosphatase (ER) (nmol/min/mg protein) | Alkaline phosphodiesterase (PM) (nmol/min/mg protein) |
|-----------|---------------------|--------------------------|--------------------------------------------------|--------------------------------------------------|-----------------------------------------------|--------------------------------------------------|
| a         | 0.1 ± 0.1           | 0.3 ± 0.0                | [3.3] 2.5 ± 0.6                                  | 1.7 ± 1.7                                        | 0.1 ± 0.1                                     | 0.1 ± 0.0                                         |
| b         | 0.7 ± 0.1           | 2.1 ± 0.3                | 54.8 ± 8.1 [71.4]                                | 55.6 ± 2.5                                       | 0.6 ± 0.1                                     | 1.3 ± 0.2                                         |
| c         | 7.4 ± 0.1           | 12.4 ± 2.7               | 9.1 ± 1.3 [8.3]                                  | 13.7 ± 6.1                                       | 228 ± 3.0                                     | 4.3 ± 0.5                                         |
| d         | 28.7 ± 2.5          | 826.2 ± 28               | 21.8 ± 5.5 [5.4]                                 | 18.4 ± 1.5                                       | 733 ± 3.5                                     | 93.0 ± 0.7                                        |
| e         | 62.9 ± 2.7          | 28.7 ± 2.7               | 12.4 ± 2.7                                       | 13.7 ± 6.1                                       | 228 ± 3.0                                     | 4.3 ± 0.5                                         |

*In order to detect [3H]hexanoyl SM synthesis in each fraction, equal volumes of the samples (equivalent to the volume of the Golgi apparatus fraction containing 25 μg of protein) were incubated with 1.0 nmol of [3H]hexanoyl Cer for 10 min at 37 °C, in a total volume of 500 μl of TK buffer. Under these conditions, for fractions c, d, e, and the homogenate, the amount of material in the incubation mixture substantially reduced the concentration of available substrate, presumably by partitioning of [3H]hexanoyl Cer into excess lipid and/or protein. This was verified by the observation that the amount of [3H]hexanoyl SM synthesis in the Golgi apparatus fraction decreased when it was incubated at constant volume in the presence of increasing amounts of fractions c, d, e, or homogenate, or in the presence of unilamellar liposomes, presumably due to partitioning of [3H]hexanoyl Cer into the membranes. To account for this effect, [3H]hexanoyl SM synthesis for a given fraction was corrected by using the decrease in the amount of [3H]hexanoy SM synthesis in the Golgi apparatus fraction (fraction b) incubated in the presence of an equivalent amount of material from the given fraction (decreased Golgi activity). For example, the corrected amount of [3H]hexanoyl SM synthesis in fraction e was calculated as:

\[
\text{Corrected SM synthesis in fraction e} = \frac{\text{Synthesis in fraction e}}{1 - \frac{\text{Decreased Golgi activity}}{\text{Total recovered activity}}} 
\]

A similar correction was made for fractions c and d and the homogenate. Recovery and distribution were calculated as described below both before (values in brackets) and after accounting for this effect.

b The recovery of protein, lipid phosphorous, and enzyme activities in the gradient is given by the sum of the values obtained for fractions a through e (total recovered activity) divided by the amount measured for the homogenate × 100. The percentage recoveries were: protein, 106 ± 11%; lipid phosphorous, 86 ± 6%; [3H]hexanoyl SM synthesis, 157 ± 18% [287%; galactosyltransferase, 79 ± 14%; glucose-6-phosphatase, 93 ± 8%; alkaline phosphodiesterase, 102 ± 11%. The distribution of each parameter for a given fraction was calculated as the ratio of the activity in that fraction divided by the total recovered activity × 100.

In order to detect [3H]hexanoyl SM synthesis in each fraction, equal volumes of the samples (equivalent to the volume of the Golgi apparatus fraction containing 25 μg of protein) were incubated with 1.0 nmol of [3H]hexanoyl Cer for 10 min at 37 °C, in a total volume of 500 μl of TK buffer. Under these conditions, for fractions c, d, e, and the homogenate, the amount of material in the incubation mixture substantially reduced the concentration of available substrate, presumably by partitioning of [3H]hexanoyl Cer into excess lipid and/or protein. This was verified by the observation that the amount of [3H]hexanoyl SM synthesis in the Golgi apparatus fraction decreased when it was incubated at constant volume in the presence of increasing amounts of fractions c, d, e, or homogenate, or in the presence of unilamellar liposomes, presumably due to partitioning of [3H]hexanoyl Cer into the membranes. To account for this effect, [3H]hexanoyl SM synthesis for a given fraction was corrected by using the decrease in the amount of [3H]hexanoy SM synthesis in the Golgi apparatus fraction (fraction b) incubated in the presence of an equivalent amount of material from the given fraction (decreased Golgi activity). For example, the corrected amount of [3H]hexanoyl SM synthesis in fraction e was calculated as:

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A similar correction was made for fractions c and d and the homogenate. Recovery and distribution were calculated as described below both before (values in brackets) and after accounting for this effect.

The distribution of marker enzymes, protein, and lipid phosphorous was determined (Table II). The Golgi apparatus fraction was almost totally devoid of marker enzymes for other subcellular compartments, indicating little cross-contamination by other membranes. The majority (56%) of recovered galactosyltransferase activity was in the Golgi appa-
ratus fraction, comparable to the recovery of $[^{14}]$C-hexanoyl SM synthesis (55%) in this fraction. Galactosyltransferase activity was enriched 58-fold in the Golgi apparatus fraction relative to the homogenate and $[^{14}]$C-hexanoyl SM synthesis was enriched 98-fold. Significant synthesis of $[^{13}]$C-hexanoyl SM was detected in fraction e, but this activity was probably due to Golgi apparatus membranes as 18% of the recovered galactosyltransferase activity was found in fraction e in addition to the majority of the recovered alkaline phosphodiesterase and glucose-6-phosphatase activities (Table II).

$[^{14}]$C-Hexanoyl SM Synthesis in Subcellular Compartments of Rat Liver—To determine the contribution of various subcellular compartments to SM synthesis in rat liver, $[^{14}]$C-hexanoyl SM synthesis was compared in enriched Golgi apparatus, PM, and ER fractions. An enriched PM fraction was prepared (29) without $Mg^{2+}$. Marker enzyme recovery (Table III) was similar to that reported (29) with alkaline phosphodiesterase enriched by 12-30-fold. Small but variable amounts of galactosyltransferase were detected (Table III) and electron microscopy revealed the presence of some Golgi apparatus cisternae. Relatively large amounts of galactosyltransferase and alkaline phosphodiesterase were observed in smooth microsomes prepared by two methods (30, 31). Rough microsome fractions were considerably purer than the smooth microsome fractions, and rough microsomes prepared according to Adelman et al. (31) showed similar marker enzyme distribution (Table III) and appeared similar by electron microscopy to that reported (31), with glucose-6-phosphatase enriched by 2.5-3-fold. Electron microscopy of the rough microsome fraction also revealed the presence of some Golgi apparatus cisternae.

$[^{14}]$C-Hexanoyl SM synthesis was compared in the enriched fractions and in a homogenate as a function of protein concentration (Fig. 3, A and D), time (Fig. 3, B and E) and substrate concentration (Fig. 3, C and F). Standard incubation conditions were chosen for each fraction so that the rate of formation of $[^{14}]$C-hexanoyl SM was linear with respect to time and protein concentration and was not limited by $[^{14}]$C-hexanoyl Cer. However, it should be noted that the rate of $[^{14}]$C-hexanoyl SM synthesis in the Golgi apparatus fraction was independent of the aqueous volume of the reaction mixture when a constant amount of $[^{14}]$C-hexanoyl Cer was added (not shown). This indicates that the $V_{\text{max}}$ of $[^{14}]$C-hexanoyl SM synthesis depends upon the partitioning of $[^{14}]$C-hexanoyl Cer into the membranes and not on the concentration of $[^{14}]$C-hexanoyl Cer in the aqueous reaction mixture. It is assumed that the formation of $[^{14}]$C-hexanoyl SM was not limited by the availability of phosphatidylcholine due to the high concentration and wide distribution of phosphatidylcholine within cellular membrane.

Small but variable amounts of $[^{14}]$C-hexanoyl SM were synthesized by PM but much less was synthesized by rough microsomes (Table III); $[^{14}]$C-hexanoyl SM synthesis was not measured in smooth microsomes due to their relatively low purity and high contamination with galactosyltransferase. Some of the $[^{14}]$C-hexanoyl SM synthesized in the PM and all

### Table III

| Fraction   | Protein (mg) | $[^{14}]$C-Hexanoyl SM synthesis | Galactosyl- | Glucose-6- | Alkaline- |
|------------|--------------|----------------------------------|-------------|-----------|-----------|
|            |              |                                  | transferase | phosphatase | phosphodiesterase |
| Golgi      | 0.6 ± 0.1    | 49.3 ± 4.7                       | 48.0 ± 5.0  | 0.6 ± 0.2  | 0.8 ± 0.1 |
| PM         | 0.7 ± 0.2    | 3.2 ± 2.5                        | 1.7 ± 1.3   | 0.8 ± 0.3  | 11.1 ± 3.3 |
| RM         | 0.1 ± 0.2    | 3.1                              | 5.0         | 10.7       | 3.5       |

$n = 3. \quad n = 6. \quad n = 2.$
vesicles were permeabilized. After dialysis against water, a

trations which permeabilized the Golgi apparatus. The other

assume

or a fluorescent analog of Cer, N-16-[{(7-nitrobenzo-2-oxa-1,3-diazol-

4-yl)amino]caproylj-D-erythro-sphingosine (g-lo), as substrate (not

based on the structural similarities between these two molecules, we

synthesis using either N-{6-[(7-Nitrobenzo-2-oxa-1,3-diazol-4-

D-erythro-sphingosine undergoes rapid transbilayer movement in Ii-

derived from subcellular fractions. Since there is no difference in SM

ment, permitting examination of the topology of SM synthesis by

addition of ["Clhexanoyl Cer to intact Golgi apparatus vesicles.

There was no difference in the topology or the amount of SM

activity in Golgi apparatus vesicles either harvested directly from the gradient or subse-

чивement in the permeabilized vesicles, compared to untreated

controls, indicating that the enzyme responsible for SM syn-

thesis was associated with a high speed pellet.

4 There was no difference in the topology or the amount of SM

synthesis in Golgi apparatus vesicles using either [14C]hexanoyl Cer or a fluorescent analog of Cer, N-6-[(7-nitrobenzo-2-oxa-1,3-diazol-

4-yl)amino]caproylj-D-erythro-sphingosine (8, 10), as substrate (not shown). N-6-[(7-Nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]caproylj-D-erythro-sphingosine undergoes rapid transbilayer movement in li-

posomes (43) and cells (6, 9, 43), and presumably also in membranes
derived from subcellular fractions. Since there is no difference in SM

synthesis using either N-6-[(7-Nitrobenzo-2-oxa-1,3-diazol-4-

yl)amino]caproylj-D-erythro-sphingosine or [14C]hexanoyl Cer, and

based on the structural similarities between these two molecules, we

assume that [14C]hexanoyl Cer also undergoes transbilayer move-

ment, permitting examination of the topology of SM synthesis by

addition of [14C]hexanoyl Cer to intact Golgi apparatus vesicles.

The specific activity is that of [14C]hexanoyl SM synthesis and % marker is the percentage of the marker enzyme

recovered in the fraction (Table III).

Total organelle activity − [1% galactosyl transferase/ % marker] × total Golgi activity, where total organelle activity and total Golgi activity are calculated as in Footnote b, % galactosyl transferase is the percentage recovered
galactosyltransferase activity per fraction and % marker is the percentage recovered marker enzyme per fraction.

4 (Activity in fraction/recovered activity) × 100, where the recovered activity is the sum of the total corrected

organelle activities in the Golgi, PM, and rough microsome fractions.

n = 11. n = 3. n = 6. n = 2.

of the [14C]hexanoyl SM synthesized in the rough microsome

efraction could be accounted for by cross-contaminating Golgi

apparatus membranes; a clear correlation was seen between the

amount of galactosyltransferase recovered in a given PM

preparation and the recovery of [14C]hexanoyl SM synthesis

in that preparation. After taking the contamination by Golgi

apparatus membranes into account, less than 13% of the [14C]

hexanoyl SM synthesized in liver could be attributed to PM

with the remainder attributed to the Golgi apparatus (Table IV).

Topography of [14C]Hexanoyl SM Synthesis in the Intact Golgi

Apparatus Fraction—The topology of the enzyme responsible

for SM synthesis was determined by examining the sensitivity of

[14C]hexanoyl SM synthesis to trypsin in intact and permea-

bilized Golgi apparatus vesicles. In intact Golgi apparatus

vesicles either harvested directly from the gradient or subse-

sequently dialyzed against 0.25 M sucrose, trypsin had no sig-

nificant effect on [14C]hexanoyl SM synthesis or galactosyl-

transferase activity (32) (Table V). A number of methods were

attempted to permeabilize the Golgi apparatus including ad-

dition of detergents, freeze-thaw, dialysis against either 10

mM NaHCO3 (pH 11) followed by dialysis against 5 mM Tris

(pH 7.4), or dialysis against 5 mM Tris (pH 7.4) alone. Detergents
could not be used as those tested (Triton X 100, sodium taurodeoxycholate, N-octyl-a-D-glucopyranoside) completely inhibited [14C]hexanoyl SM synthesis at concentra-
tions which permeabilized the Golgi apparatus. The other

methods did not render all of the galactosyltransferase activity

sensitive to trypsin. However, dialysis against water was found
to render over 95% of the galactosyltransferase activity sen-
tive to trypsin, demonstrating that the Golgi apparatus

vesicles were permeabilized. After dialysis against water, a

decrease in 30–70% of the activity of [14C]hexanoyl SM syn-

thesis was observed, presumably due to the lability of the

enzyme in the absence of salt. However, 94% of the remaining

[14C]hexanoyl SM synthesis was sensitive to trypsin treat-

ment in the permeabilized vesicles, compared to untreated

controls, indicating that the enzyme responsible for SM syn-

thesis was located on the lumenal side of the Golgi apparatus.

Furthermore, the enzyme was membrane-bound, since after cen-

trifugation (263,000 × g,, 10 min) of permeabilized Golgi

apparatus membranes, greater than 85% of [14C]hexanoyl SM

synthesis was associated with a high speed pellet.

5 The effects of other proteases on [14C]hexanoyl SM synthesis in

intact and permeabilized Golgi apparatus vesicles were examined,

however, for many of these proteases, such as Pronase and proteinase

K, no known specific inhibitor exists, and the addition of "nonspe-
cific" protease inhibitors alone inhibited [14C]hexanoyl SM synthesis. The
effect of chymotrypsin was examined without using an inhibitor. Chymotrypsin had no effect on either [14C]hexanoyl SM synthesis or galactosyltransferase activity in intact Golgi apparatus vesicles, but

completely abolished both of these activities in permeabilized vesicles.
of the contaminating alkaline phosphodiesterase activity was in  
G₀. In agreement with previous reports (45, 46) the highest 
specific activity of galactosyltransferase and the highest 
specific activity of sialyltransferase determined by immunoblot-
ting were in G₁ (Table VI), confirming that G₁ is enriched in 
trans Golgi elements (26, 28, 44, 45, 47), even though consid-
erable amounts of each were present in G₀ and G₁. 
To further characterize the Golgi subfractions, we deter-
ned the distribution of a cis/medial Golgi marker, mannos-
idase II (21, 48), by immunoblotting. In contrast to the trans 
Golgi markers, the highest specific activity of sialyltransferase II 
was in G₀ and G₁ (Table VI), but was 60% lower in G₀. In 
some experiments, mannosidase II was barely detectable in 
G₁ (not shown). 
The specific activity of [¹⁴C]hexanoyl SM synthesis paral-
leled that of mannosidase II and was 2-3 times higher in Gu 
than in G₁ (Table VI) and in some experiments there 
was no detectable synthesis of [¹⁴C]hexanoyl SM in G₀. These 
results strongly suggest that cis and medial cisternae of the 
Golgi apparatus are enriched in SM synthesis but that little 
or no synthesis of [¹⁴C]hexanoyl SM occurs in the trans Golgi 
in rat liver.

DISCUSSION

The Golgi Apparatus Is the Major Site of SM Synthesis—
In this paper we demonstrate that the Golgi apparatus is the 
major site of SM synthesis in highly enriched and well char-
acterized subcellular fractions from rat liver. In addition, we 
show that SM synthesis occurs on the lumenal side of the 
cis and medial cisternae of the Golgi apparatus. These results are 
in agreement with previous studies implicating the Golgi 
apparatus as the site of SM synthesis based on the metab-
olism, translocation, and intracellular distribution of a fluores-
cent analog of Cer in cultured fibroblasts (8-11) and in Madin-
Darby canine kidney cells (12). However, they are in dis-
agreement with earlier suggestions that the PM or ER is the 
major site of SM synthesis.

Several earlier studies implicated the PM as the major site 
of SM synthesis (1-5). However, the conclusions from these 
studies need reevaluating in light of the following: (i) Limita-
tions of subcellular fractionation in cultured cells. It is 
difficult to obtain highly purified subcellular fractions from 
cultured cells (17) and PM fractions may be contaminated 
with membranes derived from other organelles. (ii) Inade-
quate characterization of the subcellular fractions. In the 
study which established phosphatidylcholine as the proximal 
donor of the phosphorylcholine moiety in the pathway of SM 
synthesis (1) significant amounts of SM were synthesized by 
a PM fraction. However, this fraction was characterized only 
on the basis of 5'-nucleotidase activity, which is known to be 
present in both Golgi and PM fractions (48, 50). In several 
studies, the purity of the PM fractions and the contamination 
by Golgi apparatus membranes was not assessed (1-4) but in 
a study in which PM fractions were characterized for Golgi 
apparatus contamination, SM synthesis was found in a 
"Golgi-PM" fraction (6). (iii) Hydrolysis of SM at the PM. 
The effect of PM-associated neutral sphingomyelinase (18- 
20) in degrading newly synthesized SM was not evaluated in 
any of the earlier studies (1-5) and thus the quantification 
of SM synthesis could not be accurately determined. In the 
current study, neutral sphingomyelinase activity was inhibit-
ted and the PM fraction used was well characterized and 
highly enriched for a defined PM marker (Table III). After 
rectifying for contamination by Golgi apparatus membranes 
(Table IV), a small amount of SM synthesis was associated 
with the PM fraction. At present we cannot estimate the 
contribution that this small amount of SM synthesis makes 
to the total SM pool at the PM since the rate of turnover 
of SM at the PM of rat liver is not known.

The ER has also been suggested as the intracellular site of 
SM synthesis in subcellular fractions from rat (6) and mouse 
(7) liver. However, the conclusions from these studies also 
need reevaluating due to the following: (i) Incorrect choice of 
substrates. In a study using rat liver (6), a nonnaturally 
occuring stereoisomer of Cer was used. Furthermore, CDP-
choline was used as the phosphorylcholine donor, but it was 
subsequently shown (1) that CDP-choline is not involved in 
the pathway of SM synthesis. (ii) Inadequate characterization 
of the microsomal fraction. Using mouse liver (7), no attempt 
was made to differentiate between activity due to ER and 
Golgi apparatus membranes in the microsomal fractions. In 
our study, all of the SM synthesis in a rough microsome 
fraction could be accounted for by contaminating Golgi ap-
paratus membranes.

We conclude that the Golgi apparatus is the major site 
of SM synthesis in rat liver, although the possibility that other 
intracellular sites (e.g. PM or ER) contribute to the total SM 
pool in other cell types remains to be established. In addition, 
the effects of other parameters (e.g. levels of endogenous 
substrates, see Ref. 5) may need to be more fully evaluated.

The finding that the Golgi apparatus is the major site 
of SM synthesis is of particular importance in light of recent 
suggestions that the synthesis and degradation of SM may be 
coordinate regulated and that the products of SM metab-
olism may be involved in modulating the activity of protein 
kinase C (14-16). The suggestion of a coordinated role of the 
products of SM metabolism was based upon the assumption 
that most intracellular SM is synthesized at the PM (15), not 
at the Golgi apparatus. However, a subspecies of protein 
kine C has recently been found to be associated primarily 
with the Golgi apparatus in some cells (13) raising the intrigu-
ning possibility that SM synthesis may be involved in the 
regulation of protein kinase C at this organelle.

Topography and Distribution of SM Synthesis within the Golgi 
Apparatus—In contrast to some other enzymes involved in 
lipid synthesis, which are localized to the cytosolic side of 
the Golgi apparatus (53), the transferase responsible for SM 
synthesis was localized to the lumenal membranes of this 
organelle (Table V). This is similar to the localization of a 
number of glycoprotein sugar transferases (32, 51, 52) and 
glycolipid sugar transferases (53). As previously suggested 
(16), the localization of SM synthesis on the lumenal side of 
the Golgi apparatus is consistent with the fact that the ma-
jority of SM is on the external leaflet of the PM (54, 55), 
which is topologically equivalent to the lumenal leaflet of the

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**Table VI**

| Fraction | [¹⁴C]Hexanoyl SM synthesis | Mannosidase II | Galactosyltransferase | Sialyltransferase |
|----------|---------------------------|---------------|----------------------|------------------|
|          | [cis/medial] | [trans] | [cis/medial] | [trans] |
| G₀       | 0.34 ± 0.24 | 0.39 ± 0.21 | 1.31 ± 0.23 | 1.70 ± 0.65 |
| G₁       | 0.72 ± 0.39 | 0.96 ± 0.57 | 1.11 ± 0.08 | 1.36 ± 0.17 |
| G₂       | 1.00         | 1.00         | 1.00            | 1.00             |

* n = 5.  ** n = 4.
membranes of the Golgi apparatus.

While previous studies have used Golgi apparatus subfractions to examine the distribution of some phospholipids and to examine the synthesis of phosphatidylcholine (44, 56), the present study represents the first attempt to examine SM synthesis in Golgi apparatus subfractions. The procedure used for the preparation of these subfractions (28) takes advantage of the density shifts imparted to Golgi elements by enclosed lipoprotein particles. Due to the unavailability of a cis/medium Golgi apparatus marker until recently (21), the assignment of the Golgi apparatus subfractions as cis-enriched (Gc), medium-enriched (Gm), and trans-enriched (Gl) was based upon morphological criteria (28), the sequential progression of secreted proteins through the fractions (47), and upon the distribution (27, 44-46) of the terminal glycosyltransferases, sialyl-, galactosyl-, and GlcNAc-transferase, enzymes that reside in the trans Golgi (57, 58). We have now confirmed these assignments by immunoblotting with cis/medium (mannosidase II, Ref. 48) and trans (sialyltransferase, Ref. 58) Golgi apparatus markers. We found that Gl is trans-enriched but does contain small amounts of contamination by cis/medium elements.

Using these subfractions, we found enrichment of SM synthesis in cis/medium elements of the Golgi apparatus (Table VI). This result is consistent with earlier observations (9) that monensin, an inhibitor of glycoprotein transport between medial and trans regions of the Golgi apparatus (59, 60), blocks the appearance of newly synthesized fluorescent SM at the PM without inhibiting its synthesis from a fluorescent Cer precursor. We cannot exclude the possibility that the trans-enriched subfraction of the rat liver Golgi apparatus represents a subset of trans elements as the yield of trans Golgi markers obtained in this fraction is rather low but this seems unlikely due to the high specific activity of the trans Golgi subfraction in this fraction (Table VI).

We are currently attempting to isolate the enzyme responsible for SM synthesis in rat liver, phosphatidylcholine:Cer cholinephosphotransferase, obtain antibodies, and subsequently examine the distribution of the enzyme in the Golgi apparatus by immunolocalization and electron microscopy. Isolation of this enzyme would provide a means for further studying the role of the Golgi apparatus in sphingolipid synthesis, regulation, and transport.

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Note Added in Proof—In a recent report (Jeckel, D., Karrenbauer, A., Birx, R., Schmidt, R. R., and Wieland, F. (1990) FEBS Lett. 261, 155-157) SM synthesis has been examined in rat liver fractions using a truncated Cer analog with eight carbon atoms in both the long chain base and the fatty acid. Synthesis of truncated SM was found in an early (cis) compartment of the Golgi apparatus.
Sphingomyelin synthesis in rat liver occurs predominantly at the cis and medial cisternae of the Golgi apparatus.
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