

# Depletion of plasma-membrane sphingomyelin rapidly alters the distribution of cholesterol between plasma membranes and intracellular cholesterol pools in cultured fibroblasts

J. Peter SLOTTE\* and Edwin L. BIERMAN

Department of Medicine, Division of Metabolism, Endocrinology, and Nutrition, University of Washington, Seattle, WA 98195, U.S.A.

This study examines the relationship between cellular sphingomyelin content and the distribution of unesterified cholesterol between the plasma-membrane pool and the putative intracellular regulatory pool. The sphingomyelin content of cultured human skin fibroblasts was reduced by treatment of intact cells with extracellularly added neutral sphingomyelinase, and subsequent changes in the activities of cholesterol-metabolizing enzymes were determined. Exposure of fibroblasts to 0.1 unit of sphingomyelinase/ml for 60 min led to the depletion of more than 90% of the cellular sphingomyelin, as determined from total lipid extracts. In a time-course study, it was found that within 10 min of the addition of sphingomyelinase to cells, a dramatic increase in acyl-CoA:cholesterol acyltransferase activity could be observed, whether measured from the appearance of plasma membrane-derived [<sup>3</sup>H]cholesterol or exogenously added [<sup>14</sup>C]oleic acid, in cellular cholesteryl esters. In addition, the cholesteryl ester mass was significantly higher in sphingomyelin-depleted fibroblasts at 3 h after exposure to sphingomyelinase compared with that in untreated fibroblasts [ $7.1 \pm 0.4$  nmol of cholesterol/mg equivalents of esterified cholesterol compared with  $4.2 \pm 0.1$  nmol of cholesterol/mg equivalents of cholesteryl ester in control cells ( $P < 0.05$ )]. The sphingomyelin-depleted cells also showed a reduction in the rate of endogenous synthesis of cholesterol, as measured by incorporation of sodium [<sup>14</sup>C]acetate into [<sup>14</sup>C]cholesterol. These results are consistent with a rapid movement of cholesterol from sphingomyelin-depleted plasma membranes to the putative intracellular regulatory pool of cholesterol. This mass movement of cholesterol away from the plasma membranes presumably resulted from a decreased capacity of the plasma membranes to solubilize cholesterol, since sphingomyelin-depleted cells also had a decreased capacity to incorporate nanomolar amounts of [<sup>3</sup>H]cholesterol from the extracellular medium, as compared with control cells. These findings confirm previous assumptions that the membrane sphingomyelin content is an important determinant of the overall distribution of cholesterol within intact cells.

## INTRODUCTION

Unesterified cholesterol is an essential structural component of membranes in living cells. Its distribution among different membrane structures is, however, not uniform. Cholesterol is relatively abundant in plasma membranes, whereas the concentration is lower in endoplasmic reticulum and lower still in mitochondrial membranes (Ashworth & Green, 1970; Keenan & Moore, 1970; Jain, 1975; Schroeder *et al.*, 1976). It is generally believed that the phospholipid composition of a membrane structure is a major determinant of the association of cholesterol with that particular compartment. The partitioning and exchange of cholesterol between membrane structures (both biological and artificial), or among isolated cell organelles, is greatly affected by the polar-head-group composition of the membrane phospholipids (Wattenberg & Silbert, 1983; Clejan & Bittman, 1984a, 1984b) and by the phospholipid acyl chain length and saturation (Demel *et al.*, 1972; Bloj & Zilversmith, 1977; Fugler *et al.*, 1985; Child *et al.*, 1985). Interestingly, membranes with a high content of sphingomyelin and/or saturated phospholipid acyl

chains (e.g. plasma membranes) also contain a relatively high concentration of cholesterol (Colbeau *et al.*, 1971; Comte *et al.*, 1976; Schroeder *et al.*, 1976). On the other hand, membranes high in polyunsaturated phospholipids and low in sphingomyelin (e.g. mitochondria), contain very low concentrations of cholesterol. Hence, sphingomyelin as well as saturated phosphatidylcholines are believed to have higher affinities for cholesterol than unsaturated phosphatidylcholines (Demel *et al.*, 1977; Wattenberg & Silbert, 1983; van Blitterswijk *et al.*, 1987).

Previous studies from this laboratory have shown that incorporation of exogenous sphingomyelin into cultured fibroblasts results in a marked redistribution of cholesterol within the cells, as indicated by the increased rate of cholesterol biosynthesis observed in such cells (Gatt & Bierman, 1980; Kudchodkar *et al.*, 1983). In this study the effects of sphingomyelin depletion on the redistribution of plasma membrane-derived cholesterol within intact cells were tested. Results indicate that depletion of plasma membrane sphingomyelin (by the action of exogenously added sphingomyelinase) led to a rapid flow of cell surface cholesterol to the putative

Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase; DMEM, Dulbecco's modified Eagle medium.

\* Corresponding author, present address: Department of Biochemistry, ÅBO Akademi, SF-20500 Turku, Finland.

intracellular regulatory pool of cholesterol, as evidenced by the increased activity of acyl-CoA:cholesterol acyl-transferase (ACAT) and by the decreased incorporation of sodium [ $^{14}\text{C}$ ]acetate into newly synthesized [ $^{14}\text{C}$ ]cholesterol.

## EXPERIMENTAL PROCEDURES

### Materials

[ $^3\text{H}$ ]Cholesterol (32.9 Ci/mmol), [ $^{14}\text{C}$ ]oleic acid (56 mCi/mmol) and sodium [ $^{14}\text{C}$ ]acetate (57 mCi/mol) were obtained from Amersham, Arlington Heights, IL, U.S.A.). Sphingomyelin (bovine brain), sphingomyelinase (Sigma No: S-8633, from *Staphylococcus aureus*, optimal activity at pH 7.5), sphingosine and phosphorylcholine, were purchased from Sigma Chemicals, St. Louis, MO, U.S.A. Compound 58-035, an ACAT-inhibitor {3-(decyldimethylsilyl)-*N*-[2-(4-methylphenyl)-1-phenylethyl]propanamide}, was generously provided by Sandoz, East Hanover, NJ, U.S.A.

### Cells

Human fibroblasts were derived from skin biopsies of healthy volunteers. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum. Cells to be used for experiments were seeded in 35 mm dishes and allowed to grow to confluency (about 7 days) with growth medium changed every other day. When confluent, the cells were pre-incubated in serum-free DMEM for 24 h before the start of any experiments.

### Incorporation of [ $^3\text{H}$ ]cholesterol into cellular plasma membranes

To label the cellular plasma membranes with [ $^3\text{H}$ ]cholesterol tracer, confluent fibroblasts, pre-treated for 24 h in serum-free DMEM, were exposed to serum-free DMEM (1 ml/dish) containing 0.5  $\mu\text{Ci}$  of [ $^3\text{H}$ ]cholesterol/ml [32.9 Ci/mmol, added from an ethanolic stock solution, final ethanol concn. 0.1% (v/v)] for 1 h at 37 °C. With this treatment, the major part of the label is associated with the cell surface and the plasma membrane (Lange & Ramos, 1983; Slotte & Bierman, 1987). The cells were then washed extensively with 5  $\times$  2 ml of phosphate-buffered saline (pH 7.4) and used for experiments.

### Esterification of plasma membrane-derived [ $^3\text{H}$ ]cholesterol

Fibroblasts labelled with [ $^3\text{H}$ ]cholesterol in the plasma membrane were incubated with or without sphingomyelinase (0.1 unit) in 1 ml of serum-free DMEM/dish for different periods of time (0, 10–150 min). The appearance of [ $^3\text{H}$ ]cholesterol in cellular [ $^3\text{H}$ ]cholesteryl esters was determined from the cellular neutral lipid extract. In some sets of cells, esterification of [ $^3\text{H}$ ]cholesterol was determined in the presence of a specific ACAT inhibitor (compound 58-035, added in ethanol, final concn. 0.05%; control cells received solvent alone). The ACAT inhibitor was added 20 min before exposing cells to sphingomyelinase.

### [ $^{14}\text{C}$ ]Oleic acid incorporation into cellular [ $^{14}\text{C}$ ]cholesteryl esters

To measure the apparent activity of ACAT in intact cells under different incubation conditions, unlabelled cells (pre-incubated in serum-free DMEM for 24 h) were

incubated with [ $^{14}\text{C}$ ]oleic acid complexed to albumin (Oram *et al.*, 1980). Briefly, confluent fibroblasts in 35 mm dishes were incubated for 1 h at 37 °C with serum-free DMEM containing 1  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]oleic acid/ml (final concn. 20–30  $\mu\text{M}$ -oleic acid). Then, without replacement of the incubation media, 0.1 unit of sphingomyelinase was added (in 100  $\mu\text{l}$  of serum-free DMEM; control cells received serum-free DMEM alone) and the cells were exposed to the enzyme and to the [ $^{14}\text{C}$ ]oleic acid for up to 150 min. Cells were then harvested, lipids extracted, and the incorporation of [ $^{14}\text{C}$ ]oleic acid into cholesteryl [ $^{14}\text{C}$ ]oleate determined.

### Sodium [ $^{14}\text{C}$ ]acetate incorporation into endogenously synthesized cholesterol

Unlabelled, confluent fibroblasts, pre-incubated for 24 h in serum-free DMEM, were incubated with or without 0.1 unit of sphingomyelinase/ml for 150 min, as described above. To determine the apparent rate of formation *de novo* of cholesterol in these cells after the 150 min incubation, the cells were pulsed with 5  $\mu\text{Ci}$  of sodium [ $^{14}\text{C}$ ]acetate/ml for an additional 30 min (at 37 °C). The incorporation of sodium [ $^{14}\text{C}$ ]acetate into [ $^{14}\text{C}$ ]cholesterol was measured from the neutral lipid extract, as described below.

### Assay procedures

Cellular neutral lipids were extracted with hexane/isopropanol (3:2, v/v) (Brown *et al.*, 1980). Free and esterified cholesterol were separated on silica-gel H t.l.c. plates (Analtech, Newark, DE 19711, U.S.A.) with hexane/diethyl ether/glacial acetic acid (135:30:1.5, by vol.) as solvent. The cellular mass of unesterified cholesterol and cholesteryl ester mass (after alkaline hydrolysis to unesterified cholesterol) was determined by a cholesterol oxidase method (Heider & Boyett, 1978). The radioactivity in [ $^3\text{H}$ ]cholesterol, [ $^{14}\text{C}$ ]cholesterol, [ $^3\text{H}$ ]cholesteryl ester, and cholesteryl [ $^{14}\text{C}$ ]oleate spots, was determined by standard scintillation counting.

For extraction of cellular total lipids, cells were detached from the dishes by gentle scraping (teflon scraper). The cells were then pelleted by slow-speed centrifugation and the lipids were extracted by the Folch (1957) procedure. The extracted phospholipids were separated on silica-gel H plates developed in chloroform/methanol/glacial acetic acid/water (75:45:12:6, by vol.) (Skipski *et al.*, 1964). The individual phospholipid classes were quantified by the method of Bartlett (1959).

Cell proteins remaining on dishes after hexane/isopropanol extraction were digested into 1.0 ml of 0.1 M-NaOH (1 h at room temperature). A portion was taken for protein determination by the method of Lowry *et al.* (1951) with bovine albumin as standard.

## RESULTS

### Effects of sphingomyelinase treatment on cellular lipid composition

Confluent human skin fibroblasts, pre-incubated in serum-free DMEM for 24 h, were exposed to 0.1 unit of sphingomyelinase for 1 h at 37 °C. This treatment dramatically and specifically decreased the mass of sphingomyelin in the cell total-lipid extract (Table 1). The amounts of phosphatidylcholine, phosphatidylinositol, or phosphatidylethanolamine were not altered

**Table 1. Effect of sphingomyelinase treatment on cell lipid composition**

Fibroblasts were cultured in DMEM with 10% fetal calf serum. The confluent fibroblasts were then preincubated in serum-free DMEM for 24 h before exposure to sphingomyelinase. Washed cells ( $3 \times 10^6$  ml of phosphate-buffered saline, pH 7.4) in dishes (150 mm diameter) were then incubated for 60 min at 37 °C with or without 0.1 unit of sphingomyelinase/ml (0.1 unit of enzyme/210  $\mu$ g of cell protein). Lipid analysis of the cell total-lipid extract was performed as described in the Experimental procedures section. Values are averages from duplicate dishes.

Composition	Control cells	Sphingomyelinase-treated cells
Protein (mg/dish)	2.7	2.7
Lipid (nmol/mg of protein)		
Total cholesterol	88	90
Total phospholipids	267	221
Phosphatidylcholine	117	118
Sphingomyelin	13	< 1
Phosphatidylinositol	28	28
Phosphatidylethanolamine	64	68

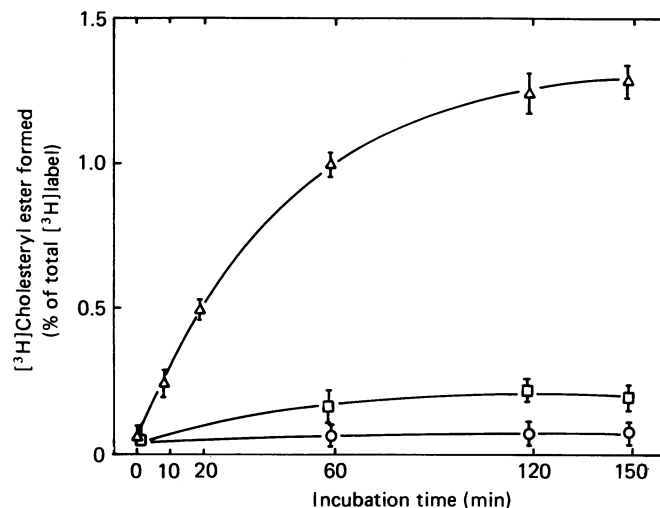
by the sphingomyelinase treatment, as compared with untreated cells. We assume that most of the sphingomyelin that was degraded by sphingomyelinase was localized in the plasma membrane, since plasma membranes normally contain most of the cell sphingomyelin (Colbeau *et al.*, 1971; Comte *et al.*, 1976; Schroeder *et al.*, 1976).

The batch of enzyme used was not contaminated by proteases, since the total amount of protein per dish was similar in treated and untreated cells. Also, the total mass of cholesterol (sum of free and esterified cholesterol) in cells was unaffected by the 1 h exposure to 0.1 unit of sphingomyelinase/ml (Table 1). The treatment of fibroblasts with 0.1 unit of sphingomyelinase/ml had only minor effects on the overall appearance of the cells, as observed by phase-contrast microscopy. Within 20 min of the onset of sphingomyelinase treatment, the cells appeared slightly rounder and swollen compared with untreated fibroblasts. However, after 60 min, both control fibroblasts and sphingomyelinase-treated cells had an indistinguishable morphological appearance.

#### Effects of sphingomyelinase treatment on esterification of plasma membrane-derived [<sup>3</sup>H]cholesterol or [<sup>14</sup>C]oleic acid

The possible effect of plasma membrane sphingomyelin depletion on the distribution of cholesterol between plasma membranes and intracellular cholesterol pools was examined by measurement of the esterification of plasma membrane-derived [<sup>3</sup>H]cholesterol. The esterification of exogenously added [<sup>3</sup>H]cholesterol in fibroblasts under the presently employed conditions is known to be catalysed by ACAT (Slotte, 1987), an enzyme localized in the RNA-rich endoplasmic reticulum (Balasubramaniam *et al.*, 1978; Hashimoto & Fogelman, 1980).

Esterification of plasma membrane-derived [<sup>3</sup>H]cholesterol in cells not exposed to sphingomyelinase



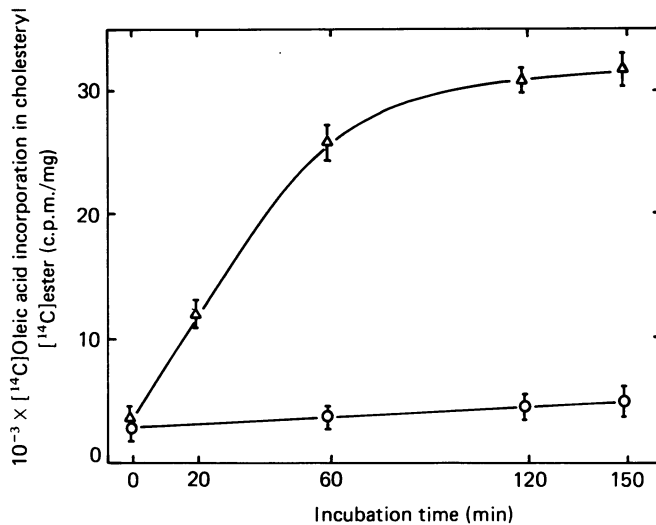
**Fig. 1. Esterification of plasma membrane-derived [<sup>3</sup>H]cholesterol in control and sphingomyelin-depleted fibroblasts**

Confluent fibroblasts in 35 mm dishes, pre-incubated for 24 h in serum-free DMEM, were exposed to 0.5  $\mu$ Ci of [<sup>3</sup>H]cholesterol/ml of serum-free DMEM for 1 h at 37 °C. After extensive rinsing ( $5 \times 2$  ml of phosphate-buffered saline), cells were incubated with ( $\Delta$ ) or without ( $\circ$ ) 0.1 unit of sphingomyelinase/ml in serum-free DMEM for up to 150 min. The esterification of plasma membrane-derived [<sup>3</sup>H]cholesterol was determined from the cellular neutral lipid extract. Cells treated with the ACAT inhibitor 58-035 (0.5  $\mu$ g/ml;  $\square$ ) were exposed to the inhibitor for 20 min before addition of sphingomyelinase. Values are given as per cent esterified [<sup>3</sup>H]cholesterol over total cell [<sup>3</sup>H]cholesterol and are means  $\pm$  S.D. of triplicate dishes from two different experiments.

was very limited (Fig. 1). This is consistent with previously reported observations under comparable incubation conditions (Lange & Matthies, 1984; Slotte & Bierman, 1987). However, when the cellular content of sphingomyelin was depleted, a dramatic increase in the esterification of plasma membrane-derived [<sup>3</sup>H]cholesterol was observed (Fig. 1), even when there was no source of exogenous cholesterol present. This effect on esterification was not seen when cells were exposed to either phosphorylcholine (at 4  $\mu$ M or 40  $\mu$ M) or sphingosine (at 1.5  $\mu$ M or 4.5  $\mu$ M) which are the water-soluble degradation products of sphingomyelin.

The cellular response to sphingomyelin depletion was very rapid, since an increased appearance of [<sup>3</sup>H]cholesterol in cellular [<sup>3</sup>H]cholesteryl esters could be detected within 10 min of addition of sphingomyelinase (Fig. 1). To confirm that the esterification of plasma membrane-derived [<sup>3</sup>H]cholesterol in sphingomyelinase-treated cells was catalysed by ACAT, sphingomyelinase-treated fibroblasts were exposed to compound 58-035 (0.5  $\mu$ g/ml) and the esterification of plasma membrane-derived [<sup>3</sup>H]cholesterol was determined. As expected, the sphingomyelinase-induced stimulation of [<sup>3</sup>H]cholesterol esterification was markedly inhibited by the ACAT inhibitor (Fig. 1), indicating that ACAT indeed was catalysing the esterification reaction in cells without the inhibitor.

In parallel to the increased esterification of plasma membrane-derived [<sup>3</sup>H]cholesterol in sphingomyelinase-



**Fig. 2. Incorporation of [<sup>14</sup>C]oleic acid into cellular cholesteryl [<sup>14</sup>C]ester in control and sphingomyelin-depleted fibroblasts**

Confluent fibroblasts, pre-incubated for 24 h in serum-free DMEM, were exposed for 1 h at 37 °C to 1  $\mu$ Ci of [<sup>14</sup>C]oleic acid/ml (20–30  $\mu$ M-oleic acid, complexed to albumin) in serum-free DMEM. Then, without replacing the incubation medium, 0.1 unit of sphingomyelinase (in 100  $\mu$ l of DMEM) was added to appropriate dishes ( $\Delta$ ). Control cells received solvent alone (DMEM;  $\circ$ ). The incorporation of [<sup>14</sup>C]oleic acid into cholesteryl [<sup>14</sup>C]ester was determined from the cellular neutral lipid extract. Values are given as means  $\pm$  S.D. of triplicate dishes from two separate experiments.

treated fibroblasts, we also observed an increased incorporation of [<sup>14</sup>C]oleic acid into cellular cholesteryl oleate (Fig. 2). Since the depletion of sphingomyelin resulted in increased esterification of both plasma membrane-derived [<sup>3</sup>H]cholesterol and exogenously added [<sup>14</sup>C]oleic acid, we think this implies that sphingomyelin depletion resulted in a redistribution of cholesterol mass within the cell, leading to a net flow of cholesterol into the endoplasmic reticulum which secondarily (and rapidly) activated ACAT.

Consistent with this interpretation was the observation that the sphingomyelinase treatment, in addition to increasing the esterification of the radioactive tracers used, also led to an increase in cell cholesteryl ester mass. In cells exposed to sphingomyelinase for 3 h in the absence of an exogenous source of cholesterol, the cholesteryl ester content was about 70% higher compared to untreated cells ( $7.1 \pm 0.4$  versus  $4.2 \pm 0.1$  nmol of sterol equivalents/mg of cell protein for sphingomyelin-depleted and control cells respectively; Table 2).

#### Effects of sphingomyelinase treatment on the rate of synthesis *de novo* of [<sup>14</sup>C]cholesterol from sodium [<sup>14</sup>C]acetate

We hypothesized that if the sphingomyelinase treatment increased the activity of ACAT by effecting a net flow of sterol mass into the endoplasmic reticulum, we should also be able to see a down-regulation of the endogenous synthesis of cholesterol after sphingomyelin depletion (Brown & Goldstein, 1980). To test this hypothesis, we measured the effect of sphingomyelinase

**Table 2. Effect of sphingomyelinase treatment on the formation of cholesteryl ester mass in cultured fibroblasts**

Confluent fibroblasts were pre-incubated for 24 h in serum-free DMEM before the experiment. Cells were then washed ( $3 \times 2$  ml of phosphate-buffered saline). Some sets of cells were exposed for 30 min to 0.1 unit of sphingomyelinase/ml in serum-free DMEM. Control cells were exposed to serum-free DMEM alone. Cells were then washed with phosphate-buffered saline ( $5 \times 2$  ml) and incubated in serum-free DMEM for 3 h at 37 °C. The cellular mass of free and esterified cholesterol was determined from the neutral lipid extract as described in the Experimental procedures section. Values are averages  $\pm$  S.D. from triplicate dishes of two separate experiments. Values marked with (\*) differ significantly from each other ( $P < 0.05$  with Student's *t* test).

Treatment	Cell cholesterol mass (nmol of sterol equivalents/mg of cell protein)		
	Free	Esterified	Total
No enzyme	85.6 $\pm$ 2.1	4.2 $\pm$ 0.1*	89.8 $\pm$ 2.2
Sphingomyelinase	84.1 $\pm$ 3.4	7.1 $\pm$ 0.4*	91.2 $\pm$ 3.8

**Table 3. Effect of sphingomyelinase treatment on the rate of cholesterol synthesis *de novo***

Confluent fibroblasts were incubated in serum-free DMEM for 24 h before the experiment. To determine the rate of endogenously synthesized cholesterol, cells were first treated with or without sphingomyelinase (0.1 unit/ml) for 150 min at 37 °C in HEPES-buffered, serum-free, DMEM. Then cells were incubated (pulsed) for an additional 30 min with 5  $\mu$ Ci of sodium [<sup>14</sup>C]acetate/ml (57 mCi/mmol). The incorporation of sodium [<sup>14</sup>C]acetate into cellular [<sup>14</sup>C]cholesterol was determined from the neutral lipid extract. Values are given as means  $\pm$  S.D. of quadruplicate dishes from one representative experiment. Values marked with (\*) differ significantly from each other ( $P < 0.05$  with Student's *t* test).

Conditions	Sodium [ <sup>14</sup> C]acetate in [ <sup>14</sup> C]cholesterol	
	(c.p.m./mg of cell protein)	(%)
Control cells	2086 $\pm$ 330*	100 $\pm$ 16
Cells + sphingomyelinase	843 $\pm$ 171*	40 $\pm$ 8

treatment on the incorporation of sodium [<sup>14</sup>C]acetate into [<sup>14</sup>C]cholesterol (Table 3). Cells which had been treated with sphingomyelinase (during a 150 min incubation in serum-free DMEM) showed a markedly reduced incorporation of sodium [<sup>14</sup>C]acetate into [<sup>14</sup>C]cholesterol compared with control cells after a 30 min pulse with the radiolabelled precursor of cholesterol. Hence it was clear that the sphingomyelinase-induced flow of cholesterol from the plasma membranes into the cells also down-regulated the formation of cholesterol *de novo*, in addition to its effect on the activity of ACAT.

### Effects of sphingomyelinase treatment on the incorporation of [<sup>3</sup>H]cholesterol into cellular plasma membranes

To test the hypothesis that sphingomyelin depletion reduced the capacity of the plasma membranes to solubilize and hence also to incorporate cholesterol, we measured how the sphingomyelinase treatment affected the ability of plasma membranes to incorporate exogenously added [<sup>3</sup>H]cholesterol. Two different labelling protocols were used. First, fibroblasts were pulsed for 1 h at 37 °C with 0.5 μCi of [<sup>3</sup>H]cholesterol/ml (15 nM). Then, after appropriate rinsing of cells with phosphate-buffered saline, the cells were exposed to 0.1 unit of sphingomyelinase/ml in serum-free DMEM for an additional 60 min. With this protocol the cellular incorporation of [<sup>3</sup>H]cholesterol was similar in control and sphingomyelinase-treated cells (Table 4), since the uptake was completed before the plasma-membrane sphingomyelin was depleted and no loss of cell-associated [<sup>3</sup>H]cholesterol occurred during the enzyme treatment. With the other labelling procedure, cells were simultaneously exposed to 0.5 μCi of [<sup>3</sup>H]cholesterol/ml and 0.1 unit of sphingomyelinase/ml. With this second procedure it was evident that cells exposed to sphingomyelinase incorporated 25% less [<sup>3</sup>H]cholesterol compared with cells not exposed to sphingomyelinase (Table 4). Hence it appears that plasma membranes depleted of sphingomyelin were able to incorporate less [<sup>3</sup>H]cholesterol than native sphingomyelin-containing membranes.

### DISCUSSION

Results in this study have demonstrated that a minor alteration in the phospholipid composition of the cellular plasma membranes markedly affects the capacity of the membrane bilayer to solubilize cholesterol, and hence affects the steady-state distribution of cholesterol between plasma membranes and other cellular organelles. Depletion of sphingomyelin from cells with neutral sphingomyelinase resulted in a rapid flow of cholesterol from the cell surface to intracellular cholesterol pools. This conclusion is supported by: (i) the increased esterification of plasma membrane-derived free [<sup>3</sup>H]cholesterol; (ii) the increased activity of ACAT, as measured by the incorporation of [<sup>14</sup>C]oleic acid into cholesteryl [<sup>14</sup>C]-oleate; (iii) the increased cellular cholesteryl ester mass; (iv) the down-regulation of cholesterol formation *de novo* in the cells, as measured by the incorporation of sodium [<sup>14</sup>C]acetate into [<sup>14</sup>C]cholesterol; (v) the reduced capacity of plasma membranes, depleted in sphingomyelin, to incorporate nanomolar amounts of exogenously added [<sup>3</sup>H]cholesterol.

These findings confirm previously obtained results from other model systems indicating that sphingomyelin has a high affinity for cholesterol and is probably a major determinant in keeping cholesterol in the plasma membrane compartment (Wattenberg & Silbert, 1983; van Blitterswijk *et al.*, 1987). It is also apparent that the plasma membranes of fibroblasts are saturated with respect to their capacity to solubilize cholesterol, since a relatively small reduction in the plasma-membrane phospholipid composition (a reduction of about 5 mol% in the total cell phospholipids) led to such a rapid mass movement of cholesterol away from the cell surface to

**Table 4. Incorporation of [<sup>3</sup>H]cholesterol into plasma membranes**

Confluent fibroblasts were incubated in serum-free DMEM for 24 h before the experiment. To label the plasma-membrane sterol pool with [<sup>3</sup>H]cholesterol, two different protocols were used. Sequential treatment: cells were first incubated for 1 h at 37 °C with 0.5 μCi of [<sup>3</sup>H]cholesterol/ml of serum-free DMEM. Then, after rinsing (5 × 2 ml of phosphate-buffered saline), cells were incubated with or without 0.1 unit of sphingomyelinase in 1 ml of serum-free DMEM (60 min at 37 °C). Simultaneous treatment: cells were incubated simultaneously with [<sup>3</sup>H]cholesterol (0.5 μCi/ml) and with or without sphingomyelinase (0.1 unit/ml) for 60 min at 37 °C. The cellular content of total [<sup>3</sup>H]cholesterol was determined from the neutral lipid extract, as described in the Experimental procedures section. Values are averages ± s.d. from triplicate dishes of one representative experiment. Values marked with (\*) differ significantly from each other (*P* < 0.05 with Student's *t* test).

Treatment	Cellular [ <sup>3</sup> H]cholesterol	
	(c.p.m./mg of protein)	(%)
Sequential treatment		
No enzyme	18030 ± 1060	100 ± 6
Sphingomyelinase	17220 ± 1145	96 ± 6
Simultaneous treatment		
No enzyme	16990 ± 1320*	100 ± 8
Sphingomyelinase	12835 ± 1075*	76 ± 6

intracellular cholesterol pools. This mass movement of cholesterol was evident from the increased incorporation of either [<sup>3</sup>H]cholesterol or [<sup>14</sup>C]oleic acid into labelled cholesteryl esters, from the increased formation of cholesteryl ester mass, as well as from the down-regulation of formation of cholesterol *de novo*. These different metabolic responses to the depletion of sphingomyelin argue against the possibility that degradation products of sphingomyelin caused the increased activity of ACAT. We did not measure the effects of ceramide on the esterification reaction, but the water-soluble degradation products (phosphorylcholine and sphingosine) did not by themselves stimulate the formation of cholesteryl esters.

The movement of plasma membrane-derived cholesterol to the substrate pool of ACAT was very rapid. It is plausible that the rapid increase in the cellular esterification activity was at least in part a result of more cholesterol appearing in the substrate pool of ACAT. Previous studies have suggested that ACAT, in cholesterol-depleted cells, may operate below optimal substrate saturation (Nilsson, 1975; Mathur *et al.*, 1981; Mitropoulos *et al.*, 1984). If that were the case in these cells, then one would expect to see a rapid increase in the activity of the ACAT reaction if there was a net flow of cholesterol mass into the substrate pool of this enzyme. However, other more complicated regulatory mechanisms could be involved in the observed up-regulation of ACAT (Hashimoto *et al.*, 1983; Chang *et al.*, 1986).

Both the up-regulation of ACAT activity and the down-regulation of the cholesterol biosynthetic pathway clearly suggest that the depletion of cell surface sphingomyelin led to a redistribution of cholesterol mass from

the cell surface into the intracellular putative regulatory pool of free cholesterol. The metabolic responses to the degradation of cell sphingomyelin in this study were exactly opposite to the corresponding responses observed in fibroblasts enriched with sphingomyelin (Gatt & Bierman, 1980; Kudchodkar *et al.*, 1983). In those studies accumulation of sphingomyelin in cultured fibroblasts led to increased synthesis of cholesterol *de novo* and to a decreased mass of cellular cholesterol esters. Hence it appears that sphingomyelin, by its ability to attract cholesterol to itself or to the microenvironment where it resides, can influence the steady-state distribution of cholesterol mass between the sphingomyelin-rich environment and other cellular environments.

Two basic conclusions can be drawn from this study. First, these results confirm previous results from other studies that sphingomyelin is an important determinant of the distribution of cholesterol within intact cells. Secondly, it is obvious that plasma-membrane cholesterol has the potential to be rapidly transported from the cell surface to intracellular cholesterol pools, whenever the cholesterol-solubilizing capacity of the plasma membranes is reduced. This is an important finding, since it shows that plasma-membrane cholesterol is not metabolically isolated from the putative intracellular regulatory pool of cholesterol.

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