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Different metabolic recycling of the lipid components of exogenous sulphatide in human fibroblasts

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Cultured human fibroblasts were fed with two differently labelled sulphatide molecules [one labelled on C-3 of the sphingosine (Sph) moiety ([*Sph*-^{3}H]sulphatide), the second on C-1 of stearic acid ([*stearoyl*-^{14}C]sulphatide)], and the intracellular metabolic fate of radioactivity was monitored. Incorporated radioactivity was almost all recovered in the total lipid extract, regardless of the labelling position of the added sulphatide; however, large differences in the level of incorporation occurred among labelled glycosphingolipids. For example, sphingomyelin was present as the major radiolabelled lipid after [*Sph*-^{3}H]-sulphatide incubation, but was detectable only in trace amounts after [*stearoyl*-^{14}C]sulphatide administration; in the latter case the radioactivity was located predominantly in glycerophospholipids. From this finding it can be inferred that the free long-chain base (sphingosine) that originates from lysosomal catabolism of sulphatide is mainly, and quite specifically, utilized for sphingomyelin biosynthesis, whereas the ceramide moiety is not; conversely the fatty acid released from ceramide is non-specifically re-utilized for phospholipid biosynthesis.

INTRODUCTION

For several years it has been common practice to make use of exogenous materials, administered to animals or cultured cells, in order to examine the nature of their degradation at the level of the lysosomal apparatus and to investigate the possible occurrence of salvage or recycling mechanisms. In fact, among the products which originate from intralysosomal degradative processes, some are able to cross the lysosomal membrane and be re-utilized as substrates for biosynthetic purposes; these include those formed in glycosphingolipid catabolism.

In particular, the use of glycosphingolipids radiolabelled on the C-3 position of the long-chain base (sphingosine) has provided evidence for the recycling of degradation product(s) in the biosynthesis of gangliosides and sphingomyelin (Ghidoni *et al.*, 1986). Owing to the stability of the labelling position, this (or these) product(s) of degradation or of biosynthesis should contain the long-chain base. It is not yet known whether free long-chain base alone and/or ceramide is (are) re-utilized for sphingomyelin re-synthesis or whether any of the above compounds, or even glucosylceramide, can be used for ganglioside re-synthesis. The use of exogenous glycolipids labelled at the fatty acid moiety and precursors labelled at the long-chain base enables one to check whether recycling of intact ceramide occurs.

In the present study we administered two differently labelled sulphatide molecules to cultured human fibroblasts (one ^{3}H-labelled on the C-3 position of the long-chain base, the second ^{14}C-labelled on the C-1 position of the fatty acid moiety) and compared their intracellular fates. The use of sulphatide was suggested because this lipid was reported to be extensively taken up, in the absence of added detergents, and metabolized by cultured fibroblasts (Porter *et al.*, 1971); in addition, and contrary to the situation with gangliosides, it does not interfere, as a precursor, in the biosynthesis of more complex glycosphingolipids.

EXPERIMENTAL

Materials

Sulphatide (from bovine brain) was from Sigma (St. Louis, MO, U.S.A.); 0.025% trypsin/0.02% EDTA in special salt solution, EMEM, Hank's solution and PBS were from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.); foetal-calf serum (FCS) was from NABI (Miami, FL, U.S.A.). NaB^3H_4 (6.4 Ci/mmol), [1-^{14}C]stearic acid (50 mCi/mmol) and [*N-methyl*-^{14}C]-sphingomyelin (52 mCi/mmol) were from Amersham International, Amersham, Bucks., U.K. Visking dialysis tubing [inflated diameter 6.3 mm, average pore radius 2.4 nm (24 Å)] were from the Scientific Instrument Centre, London W.C.1, U.K. Standard radiolabelled [*Gal*-^{3}H]lactosylceramide was prepared by the galactose oxidase/$NaBH_4$ method described by Leskawa *et al.* (1984); standard radiolabelled [*Sph*-^{3}H]galactosylceramide and [*Sph*-^{3}H]ceramide were prepared by the DDQ/NaB^3H_4 procedure as described by Kishimoto & Mitri (1974). The radiochemical purity of all ^{3}H-labelled compounds was better than 90%, and the specific radioactivity was in the range 0.7–1.1 Ci/mmol.

Abbreviations used: *Sph* (in labelled compounds), sphingosine; EMEM, Eagle minimal essential medium with Earle's salts; PBS, Dulbecco phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}; FCS, foetal-calf serum; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone.

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Preparation of [*Sph*-^{3}H]sulphatide

[*Sph*-^{3}H]Sulphatide was prepared by the DDQ/NaB^3H_4 method as described by Ghidoni *et al.* (1981) and modified as follows: 10 ml of a solution of sulphatide (3 mg/ml) in chloroform/methanol (2:1, v/v) were mixed with 10 ml of Triton X-100 solution (20 mg/ml) in the same solvent. After evaporation to dryness, the residue was dissolved in 10 ml of DDQ solution (0.3 g/ml in 'sodium-dehydrated' toluene). The mixture was allowed to react at 37 °C for 48 h with stirring in a screw-capped tube; purification of the oxidized sulphatide was performed as described by Ghidoni *et al.* (1981), with minor modifications. These comprised the elution of the silica-gel chromatographic column, which was carried out with 100 ml of chloroform/methanol (6:1, v/v) and thereafter with 200 ml of chloroform/methanol (1:1, v/v). The oxidized sulphatide was labelled by reduction with [^{3}H]$NaBH_4$, as previously described for other glycosphingolipids (Ghidoni *et al.*, 1981). The labelled sulphatide was purified by chromatography on a silica-gel 100 column (110 cm × 1.5 cm) equilibrated and eluted with chloroform/methanol/water (120:70:7, by vol.). The labelled sulphatide was made homogeneous in the lipid portion by, in the case of the fatty acid moiety, the de-*N*-acylation/re-*N*-stearoylation method of Dubois *et al.* (1980), and, subsequently, in the case of the long-chain-base moiety, silica-gel chromatography using chloroform/methanol/water (625:100:2, by vol.) as eluent. Only the molecular species containing stearic acid as fatty acid and *erythro*-C_{18}-sphingosine as long-chain base were used. The radiochemical purity of the latter-named molecular species of [*Sph*-^{3}H]sulphatide, assessed immediately before treatment, was 98%. The resulting specific radioactivity was 0.6 Ci/mmol.

Preparation of [*stearoyl*-^{14}C]sulphatide

[*stearoyl*-^{14}C]Sulphatide was prepared by coupling [^{14}C]stearoyl chloride to lysosulphatide. Lysosulphatide was prepared, starting with 30 mg of sulphatide, by a modification of the method (applied to ganglioside G_{M1}) of Sonnino *et al.* (1985). The modification involved a silica-gel column (100 cm × 1.5 cm) that was equilibrated and eluted with chloroform/methanol/water (120:70:7, by vol.). Preparation of [^{14}C]stearoyl chloride and its condensation with lysosulphatide were performed as described by Dubois *et al.* (1980), starting with 30 mg of sulphatide. [*stearoyl*-^{14}C]Sulphatide had a specific radioactivity of 50 mCi/mmol and a radiochemical purity, assessed by t.l.c. immediately before treatment, of 98%.

Cell-culture conditions

Skin biopsies were obtained by the punch technique from normal young individuals, and fibroblasts were cultured, initiated and maintained as described by Leroy *et al.* (1972) in 75 cm^2 Corning plastic flasks. Subcultures were made on 28 cm^2 culture dishes by using 5 ml of EMEM containing 10% (v/v) FCS. Fibroblast cultures were used at confluence (130–150 μg of cell protein/dish).

Treatment of human fibroblasts with labelled sulphatide

Samples of [*Sph*-^{3}H]sulphatide or [*stearoyl*-^{14}C]-sulphatide, dissolved in chloroform/methanol (2:1, v/v) were added to unlabelled sulphatide, and the resulting solution was pipetted into a sterile tube and dried in a stream of nitrogen. The residue was resuspended in an appropriate volume of EMEM, in the presence of 10% FCS, to obtain a final concentration of sulphatide of 5 μM (having a specific radioactivity of 0.8 μCi/ml). A 3 ml portion of the sulphatide-containing medium was added to each culture dish (100–150 μg of cell protein/dish) from which the original medium had previously been removed with great care. Incubation was prolonged for 48 h, with no change of the sulphatide-containing medium. At the end of the incubation, the medium was removed, and the cells were washed with 3 ml of Hanks solution (three times), in order to remove unbound sulphatide. Thereafter cells were treated with 2 ml of PBS solution containing 0.1% trypsin (10 min), in order to remove all the radioactivity absorbed on the cell surface and/or interacting with some membrane proteins.

Extraction, fractionation and analysis of lipids

Extraction of total lipids from the cell pellet and partition into an organic (non-gangliosidic-lipid-containing) and an aqueous (ganglioside-containing) phase were performed as described by Tettamanti *et al.* (1973). In detail, the cell pellet was homogenized in 2 ml of 0.01 M-potassium phosphate buffer/tetrahydrofuran (1:4, v/v) and centrifuged at 900 *g* for 5 min. The supernatant was collected and the pellet was extracted with buffer/tetrahydrofuran three further times. The pooled supernatants, after addition of 0.3 vol. of diethyl ether, were shaken for 2 min and then centrifuged at

Table 1. Distribution of radioactivity in the different fractions obtained from human fibroblasts after administration of sulphatide

Results are means for five experiments; S.D. values are less than 10% of mean values. Abbreviation: n.d., not detectable.

	Incorporated radioactivity			
	[*Sph*-^{3}H]Sulphatide		[*stearoyl*-^{14}C]Sulphatide	
Fraction	(d.p.m./mg of protein)	(%)	(d.p.m./mg of protein)	(%)
Whole homogenate	139890	100	111320	100
Lipid extract	138900	99.3	109670	98.5
Dried organic phase	108350	77.5	92780	83.3
Dialysed aqueous phase	n.d.	n.d.	840	0.8
Diffusible fraction	30450	21.8	16050	14.4

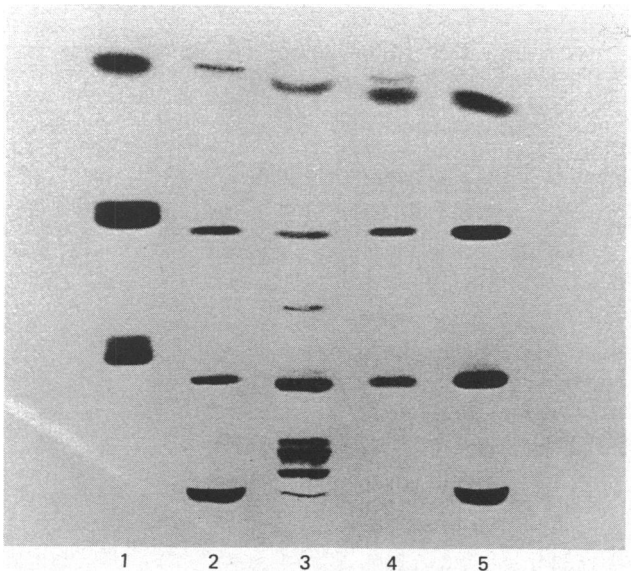

Fig. 1. Radioactivity distribution in the different lipids metabolically derived from sulphatide

The lipids were separated by t.l.c., with chloroform/methanol/water (55:20:3, by vol.) as eluting solvent system, and revealed by fluorography. Lane 1, standard radiolabelled ceramide, glucosylceramide and lactosylceramide (in order from the top); lane 2, radiolabelled lipids formed after [*Sph*-^{3}H]sulphatide administration; lane 3, radiolabelled lipids formed after [*stearoyl*-^{14}C]sulphatide administration; lane 4, alkaline hydrolysis of the lipids formed after [*stearoyl*-^{14}C]sulphatide administration; lane 5, standard radiolabelled stearic acid, galactosylceramide, sulphatide and sphingomyelin (in order from the top).

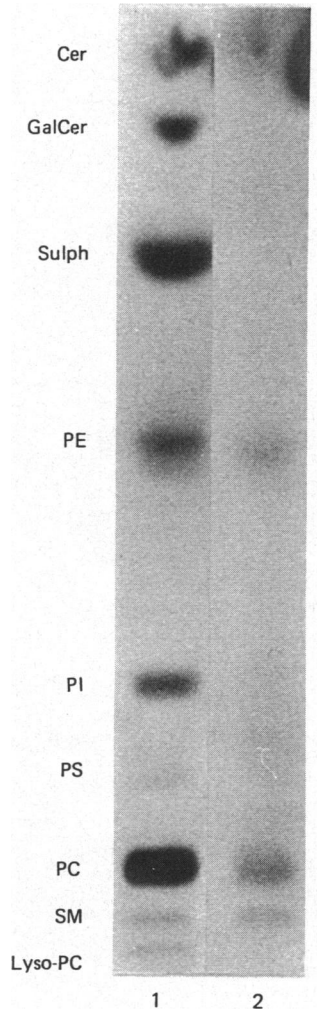

Fig. 2. Radiolabelled lipids obtained after administration of [*stearoyl*-^{14}C]sulphatide (lane 1) and endogenous fibroblast phospholipids (lane 2)

The lipids were separated by t.l.c., with chloroform/methanol/acetic acid/water (30:20:2:1, by vol.) as eluting solvent system. Lane 1, radiolabelled lipids obtained after [*stearoyl*-^{14}C]sulphatide administration; detection by fluorography. Lane 2, endogenous fibroblasts phospholipids; detection by exposure to I_2 vapour. Abbreviations: Cer, ceramide; GalCer, galactosylceramide; Sulph, sulphatide; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; Lyso-PC, lysosphosphatidylcholine.

600 *g* for 10 min. The aqueous phase was removed, and the organic phase, after addition of 0.1 vol. of distilled water, was shaken and centrifuged as described above. The newly formed aqueous phase was added to the former. The pooled aqueous phases were evaporated, resuspended in 2 ml of distilled water and dialysed for 2 days at 2–4 °C against 1 litre of distilled water, which was changed three times a day.

The lipids contained in the organic phase were separated by t.l.c., using chloroform/methanol/water (55:20:3, by vol.; optimal for sphingolipid separation) or chloroform/methanol/acetic acid/water (30:20:2:1, by vol.; optimal for phospholipid separation) as eluting solvent mixtures, and the distribution of radioactivity in individual lipids was determined by fluorography and radiochromatoscanning (Ghidoni *et al.*, 1986), radiolabelled standards being used as references. Alkaline methanolysis [0.5 M-NaOH in methanol/water (1:1, v/v) overnight at 37 °C], to assess the presence of glycerophospholipids, was performed on portions (containing ~ 10000 d.p.m.) of the dried organic phase obtained after both treatments. Characterization of other individual radiolabelled lipids was performed after purification by preparative t.l.c. For this the lipid extract, corresponding to 1 mg of cell protein, after alkaline methanolysis, was spotted on to a 20 cm × 20 cm preparative t.l.c. plate (Merck G.m.b.H., Darmstadt, Germany); the plate was eluted with chloroform/methanol/water (55:20:3, by vol.), exposed to I_2 vapour, and positive spots scraped off; the gel was washed with 5 ml of chloroform/methanol (4:1, v/v). The recovery of radioactivity was in all cases higher than 90 %. Each radiolabelled compound was identified by submitting it to chemical or enzymic analysis. In particular, the spot corresponding on t.l.c. to sphingomyelin was submitted to sphingomyelinase treatment (Ikezawa *et al.*, 1978); the spot corresponding on t.l.c. to sulphatide was submitted to acid methanolysis (anhydrous 0.05 M-HCl in methanol at 40 °C for 80 min); the spots corresponding on t.l.c. to galactosylceramide and ceramide were submitted to acid hydrolysis (2.5 M-formic acid at 100 °C for 18 h). The products formed after all these treatments were separated by t.l.c. by using the same solvent system as that described above, submitted to radiochromatographic scanning, and identi-

Table 2. Radioactivity distribution in the different lipids obtained from human fibroblasts after administration of sulphatide

Results are means for five experiments; S.D. values are less than 10% of mean values. Abbreviation: n.d., not detectable.

Fraction	Incorporated radioactivity			
	[*Sph*-^{3}H]Sulphatide		[*stearoyl*-^{14}C]Sulphatide	
	(d.p.m./mg of protein)	(%)	(d.p.m./mg of protein)	(%)
Ceramide	10730	9.0	9920	10.7
Galactosylceramide	19810	18.3	16530	17.8
Sulphatide	25910	23.9	22420	24.2
Phosphatidylcholine	n.d.	–	22640	24.3
Phosphatidylethanolamine	n.d.	–	9060	9.8
Phosphatidylinositol	n.d.	–	6550	7.1
Phosphatidylserine	n.d.	–	2810	3.0
Lysophosphatylcholine	n.d.	–	1270	1.4
Total glycerophospholipids	–	–	42330	45.6
Sphingomyelin	51900	47.9	1580	1.7
Total dried organic phase	108350	100	92780	100

fied by comparison with authentic standards of known structure.

Determination of radioactivity

Determination of radiolabelled lipids, separated by t.l.c., by fluorography or by radiochromatography, counting of radioactivity in solution and determination of radioactivity were performed as previously described (Ghidoni *et al.*, 1983).

RESULTS AND DISCUSSION

The incorporation of radioactivity into fibroblasts 2 days after addition to the media of identical amounts (5 μM final concn., 2.4 μCi) of sulphatide, ^{3}H-labelled in the long-chain base or ^{14}C-labelled in the fatty acid moiety, is reported in Table 1. Incorporated radioactivity was almost all recovered in the lipid extract, regardless of the labelling position of added sulphatide. The high value of incorporation after sulphatide administration [5–10-fold higher than that recovered after feeding of fibroblasts with other glycosphingolipids (Fishman *et al.*, 1983; Sonderfeld *et al.*, 1985; Chigorno *et al.*, 1985; Zeigler & Bach, 1986) could be explained by assuming a different uptake mechanism. The higher value of incorporation observed after incubation with [*Sph*-^{3}H]-sulphatide suggests a more extensive utilization, for biosynthetic purposes, of the free long-chain base, whereas the lower value exhibited after incubation with [*stearoyl*-^{14}C]sulphatide indicates a major involvement of the fatty acid in energy-producing processes, resulting in $^{14}CO_2$ formation. This does not exclude, however, the possible re-utilization of the fatty acyl residue for lipid biosynthesis, although only to a minor extent.

After partition in diethyl ether the radioactivity is mainly (about 80%) recovered in the dried organic phase, regardless of the labelled sulphatide administered; the remainder (about 20%) is recovered in the aqueous phase, but is almost completely lost after evaporation and dialysis, indicating that this portion of radioactivity is associated with diffusible low-molecular-mass substances, including water or CO_2.

The distribution of radioactivity in the different lipids, present in the dried organic phase, is reported in Figs. 1 and 2 and Table 2. Structural characterization, by chemical or enzymic hydrolysis, of individual radiolabelled compounds formed after administration to cells of both [*Sph*-^{3}H]sulphatide and [*stearoyl*-^{14}C[sulphatide lead to the following conclusions: (i) some spots, present only after [*stearoyl*-^{14}C]sulphatide administration, are labile to alkaline hydrolysis (Fig. 1, lanes 2–3), the radioactivity being moved to the fatty acid region. These radioactive spots co-migrate on t.l.c. with endogenous fibroblast glycerophospholipids (Fig. 2); therefore, they can be identified as authentic glycerophospholipids. Among them, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol and lysophosphatidylcholine could be recognized; (ii) four main compounds, present after both treatments, are resistant to alkaline hydrolysis (Fig. 1, lanes 2–3). These, after isolation by preparative t.l.c., behave under chemical or enzymic hydrolysis like authentic standards of known structure. So the most polar of these, affected by sphingomyelinase and yielding ceramide, is therefore identified as sphingomyelin. The second compound, in terms of polarity, is susceptible to acid methanolysis, yielding galactosylceramide, and is identified as sulphatide. The third is hydrolysed in formic acid to ceramide and is, therefore, identified as galactosylceramide. The least-polar compound remains unaffected by acids and is identified as ceramide.

By analysis of the differences in the distribution of radioactivity in individual lipids according to the type of administered sulphatide (Table 2), it was found that the residual sulphatide and its catabolites (galactosylceramide and ceramide) were present in similar amounts; in contrast, sphingomyelin was present as the major radiolabelled lipid (47.9% of total organic phase) after [*Sph*-^{3}H]sulphatide incubation, but it is detectable only in very minor amounts (1.7% of total organic phase) after

[*stearoyl*-^{14}C]sulphatide administration. In this latter case, a large amount of radioactivity (45.6% of total organic phase) is located in glycerophospholipids.

All these data reflect the different fates of the labelled parts of the sulphatide molecule: the free long-chain base is mainly, and quite specifically, utilized for sphingomyelin re-synthesis, whereas the ceramide is not; conversely, the fatty acid is recycled and non-specifically re-used for phospholipid biosynthesis; analogies with the '*de novo*' pathway of sphingomyelin biosynthesis are not possible, since the question as to whether ceramide itself is able or not able to cross the lysosomal membrane still remains and deserves further investigation.

A final point of interest concerns the absence of formation of radiolabelled gangliosides after administration of both labelled sulphatides (Table 1), even though gangliosides were formed by re-synthesis when other glycosphingolipids, different from sulphatide, were administered to animals (Ghidoni *et al.*, 1986). An explanation for this finding could be that the re-synthesis of gangliosides does not occur starting from the free long-chain base or the ceramide as precursors, but only from glucosylceramide or some other more glycosylated fragment, as alrready proposed for rat liver (Ghidoni *et al.*, 1986); alternatively, it cannot be excluded that cultured cells and living animals show different, and not comparable, behaviours.

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