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Metabolism of Sphingosine Bases, VII[1–6]

Studies on the Metabolism of [1-^{3}H]*erythro*-DL-Sphingosine ([1-^{3}H]4*t*-sphingenine) during the Myelination Period of the Rat

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Summary: [1-^{3}H]4*t*-sphingenine was administered to suckling rats during the myelination period (10th—26th postnatal day) by repeated intraperitoneal injections. The total lipids of the liver and brain were isolated and separated into their radioactive components. The following lipids were labeled: ceramide, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin. Ceramide was labeled in the long-chain base, the other sphingolipid sphingomyelin in the base and the choline moiety. Phosphatidylethanolamine and phosphatidylcholine carried the total activity in ethanolamine and choline respectively. Sphingomyelin of brain was labeled only in the choline moiety, whereas the sphingosine base was not radioactive. The ceramide fraction of brain contained only trace amounts of labeled sphingosine and the cerebroside fraction was not labeled. No free radioactive sphingosine could be detected in the brain.

These results indicate that during the myelination period intraperitoneally administered [1-^{3}H]4*t*-sphingenine does not reach the brain as an intact base and all the long chain bases of the sphingolipids in the brain must be synthesized in the glial and/or ganglion cells during the myelination period.

Our results indicate that 95.3% of the radioactivity recovered from the brain and 60.4% of that from the liver is present as ^{3}H-labeled ethanolamine or choline, incorporated into phosphatidylethanolamine and phosphatidylcholine. In the liver ceramide and sphingomyelin are also labeled in the long-chain base of the molecule. Our results give proof for an additional pathway for the formation of the ethanolamine and choline of phospholipids from serine via the degradation of long-chain sphingosine bases.

Zusammenfassung: *Stoffwechsel der Sphingosinbasen, VII: Untersuchungen über den Stoffwechsel von [1-^{3}H]erythro-*DL*-Sphingosin ([1-^{3}H]4t-Sphingenin) während der Myelinisierungsphase der Ratte.* [1-^{3}H]4*t*-Sphingenin wurde neugeborenen Ratten während der Myelinisierungsphase (10. bis 26. Lebenstag) mehrmals injiziert. Aus Leber und Gehirn wurden die Gesamtlipoide isoliert und ihre radioaktiven Komponenten getrennt. Die folgenden Lipoide waren radioaktiv: Ceramid, Kephalin, Lecithin und Sphingomyelin. Ceramid war im Sphingosinanteil markiert, während die Radioaktivität von Sphingomyelin im Sphingosin- und im Cholinanteil lokalisiert war. Die gesamte Aktivität von Kephalin und Lecithin fand sich im Äthanolamin bzw. im Cholin. Im Sphingomyelin

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[1] I. Commun.: W. Stoffel and G. Sticht, this journal **348**, 941 [1967].
[2] II. Commun.: W. Stoffel and G. Sticht, this journal **348**, 1345 [1967].
[3] III. Commun.: W. Stoffel and G. Sticht, this journal **348**, 1561 [1967].
[4] IV. Commun.: W. Stoffel, D. LeKim and G. Sticht, this journal **348**, 1570 [1967].
[5] V. Commun.: W. Stoffel, D. LeKim and G. Sticht, this journal **349**, 664 [1968].
[6] VI. Commun.: W. Stoffel, G. Sticht and D. LeKim, this journal **349**, 1149 [1968].

des Gehirns befand sich die gesamte Radioaktivität im Cholin, während sich die Sphingosinbase als inaktiv erwies. Die Ceramidfraktion des Gehirns enthielt nur Spuren des eingesetzten tritiierten Sphingosins. In den Cerebrosiden war keine Aktivität nachweisbar. Diese Ergebnisse zeigen, daß intraperitoneal appliziertes Sphingosin das Gehirn nicht erreicht. Demzufolge muß der Sphingosinanteil der Gehirnsphingolipoide während der Myelinisierungsphase in den Glia- und/oder den Ganglienzellen synthetisiert werden.

Im Gehirn lagen 95,3% und in der Leber 60,4% der Gesamtaktivität im Äthanolamin bzw. Cholin der entsprechenden Phospholipoide eingebaut vor. In Übereinstimmung mit unseren früheren Ergebnissen werden die Kohlenstoffatome 1 und 2 als Äthanolamin abgespalten, das dann durch Methylierung in Cholin übergeführt werden kann. Beide Verbindungen werden schnell in Kephalin und Lecithin übergeführt. Damit ist ein weiterer Weg für die Bildung der Äthanolamin- bzw. Cholinbase der Phospholipoide über den Abbau der Sphingosinbasen nachgewiesen.

Previous studies[1–6] on the metabolism of sphinganine (dihydrosphingosine) and 4*t*-sphingenine (sphingosine) have given conclusive evidence that sphinganine is biosynthesized from palmitoyl-CoA and L-serine to yield 3-oxosphingosine, which then is reduced by an NADPH-dependent reductase to sphinganine[5]. [3-^{14}C]sphinganine is degraded to [1-^{14}C]palmitate and [1-^{14}C]sphinganine yields ^{14}C-labeled ethanolamine and choline respectively; both were isolated in the free state and found predominantly as their phosphate esters and as the base moiety in phosphatidylcholine, phosphatidylethanolamine and sphingomyelin. [7-^{3}H]4*t*-sphingenine also yields ^{3}H-labeled palmitate. The present work reports studies on the metabolism of [1-^{3}H]4*t*-sphingenine ([1-^{3}H]*erythro*-DL-sphingosine) in the newborn rat during the 13th to the 26th postnatal day. This time interval coincides with the period of active myelination.

Early investigations on the lipid metabolism during the period of myelination indicated that a rapid increase of the synthesis of brain phospholipids occurs between the 10th and 20th day and of brain cerebrosides between the 20th and 40th day of extrauterine life[7]. ZABIN and MEAD[8] and SPRINSON and COULON[9] studied the incorporation of serine carbon atoms into the polar groups of sphingosine bases of brain sphingolipids during the myelination period after intraperitoneal administration to rats. Although this precursor was incorporated into brain sphingosine bases nothing could be said about the place of synthesis of these bases. They could be synthesized either in the liver and transported through the blood-brain barrier, or the synthesis could proceed in the brain. We therefore studied the metabolic fate of [1-^{3}H]4*t*-sphingenine in the myelination period (13th—26th day of extrauterine life) after intraperitoneal administration into rats.

Results

The studies presented in this paper were made possible after the synthesis of [1-^{3}H]4*t*-sphingenine had been achieved. We labeled this compound by the reduction of ethyl 2-amino-3-hydroxy-4*t*-octadecenoate hydrochloride with $LiAl^{3}H_4$. The labeled sphingosine proved to consist entirely of the *erythro* form of 4*t*-sphingenine as shown by gas-liquid chromatography (SE 30, 3.8% on Chromosorb column) of the *N*-acetyl-di-*O*-trimethylsilyl derivative[10].

The [1-^{3}H]4*t*-sphingenine hydrochloride was injected five times in intervals of two days intraperitoneally into young rats starting on the 13th day after birth. The brains and the livers of seven animals were pooled separately on the 26th day and the total lipids extracted. The separation of the lipid classes was carried out by silicic acid column chromatography.

Only ceramides, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin were labeled, the quantitative data are given in the Table.

Liver and brain lipids contained a minor labeled component, the structure of which remains to be determined. The chromatographically pure fractions were analyzed by enzymatic degradation with phospholipase C* in order to localize the label in the molecule.

[7] W. KOCH and M. KOCH, J. biol. Chemistry **15**, 423 [1913].

[8] J. ZABIN and J. F. MEAD, J. biol. Chemistry **205**, 271 [1953]; **211**, 87 [1954].

[9] D. B. SPRINSON and A. COULON, J. biol. Chemistry **207**, 585 [1954].

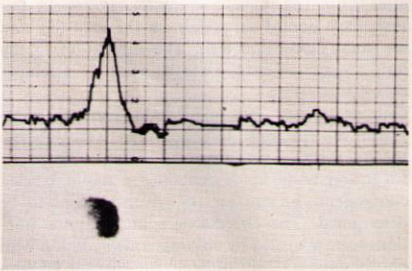
* Phospholipase C, Phosphatidylcholine cholinephosphohydrolase (EC 3.1.4.3).

[10] H. E. CARTER and R. C. GAVER, J. Lipid. Res. **8**, 391 [1967].

Table. Distribution of radioactivity in the brain and liver lipids of young rats after intraperitoneal application of [1-^{3}H]4*t*-sphingenine hydrochloride.

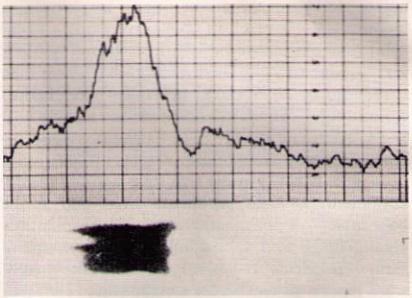
	brain		liver	
	radioactivity [dpm]	% of total activity	radioactivity [dpm]	% of total activity
total lipid	2929500	100	8784320	100
ceramide	27300	0.9	2143220	24.4
phosphatidylethanolamine	1170000	39.9	2375800	27.0
phosphatidylcholine	1615000	55.3	2938000	33.4
4*t*-sphingenine	—	—	175000	2.0
sphingomyelin	117200	3.9	1152300	13.1

After hydrolysis with phospholipase C phosphatidylethanolamine, phosphatidylcholine and sphingomyelin yielded phosphorylethanolamine and phosphorylcholine as water soluble hydrolysis products and diglycerides and ceramide, respectively, as ether soluble products. The diglycerides of the two phospholipid classes of the brain and the liver contained no radioactivity, a result which is in full accordance with our previous results derived from the [1-^{3}H] and [1-^{14}C]sphinganine experiments[2]. The total radioactivity of phosphatidylethanolamine and phosphatidylcholine was localized in the two bases ethanolamine and choline. Phosphorylethanolamine and phosphorylcholine were identified by radio-thin-layer chromatography on cellulose layers (Fig. 1, solvent system: phenol/water 9:1 for phosphorylethanolamine, and butanol/glacial acetic acid/water:40:20:20 for phosphorylcholine).



1a

Fig. 1. Radio-thin-layer chromatography (a) of phosphorylethanolamine (solvent system: phenol/water 9:1) and (b) of phosphorylcholine (solvent system: butanol/glacial acetic acid/water 40:20:20) on cellulose thin layers.



1b

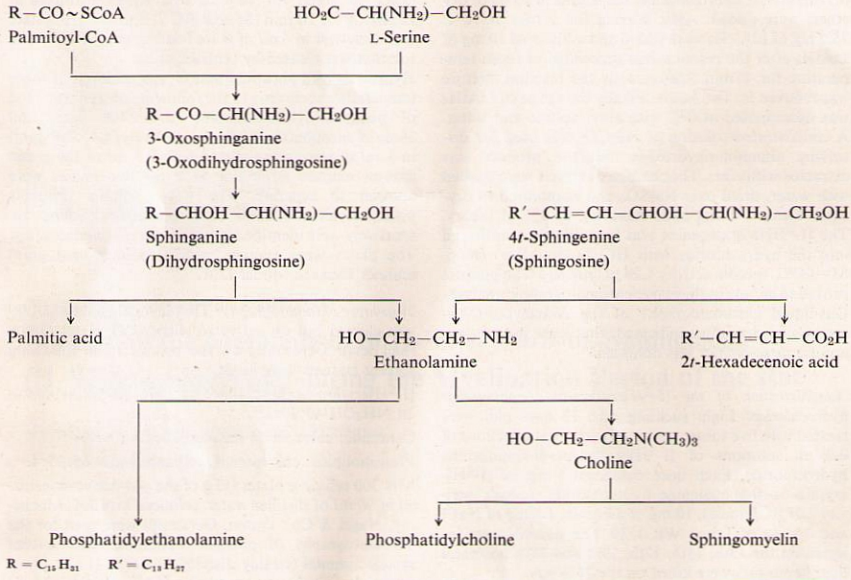
Enzymatic hydrolysis with phospholipase C of brain sphingomyelin yielded only labeled choline phosphate. Liver sphingomyelin, however, was labeled both in the long-chain base (97%) and the choline moiety (3%).

The free ceramide of the brain lipid extract contained only 0.93% of the total radioactivity, whereas the ceramide of liver was labeled in the long chain base to an extent of 24.4% of the total radioactivity.

Furthermore under the conditions of our experiments (intraperitoneal application and myelination period of the animals) we could not detect 4*t*-sphingenine as base component of sphingolipids in the brain. After absorption by the peritoneum the 4*t*-sphingenine base has to pass the liver, a way, which is similar to the physiological conditions of the enteral absorption of exogenous long-chain bases. KANFER[11] injected ^{3}H-labeled 4*t*-sphingenine intracerebrally and observed incorporation into

[11] J. N. KANFER and A. E. GAL, Biochem. biophysic. Res. Commun. **22**, 442 [1966].

Fig. 2. Transformation of serine carbon atom 2 and 3 into ethanolamine and choline of phosphatidylethanolamine and phosphatidylcholine via long chain sphingosine bases.



ceramide and sphingomyelin. No quantitative data about the incorporation into the lipids or about their metabolic fate are given. The phospholipids were not investigated.

In our experiment no [1-^{3}H]4*t*-sphingenine has reached the brain tissue. One reason might be that the continuously supplied labeled base is absorbed via the portal vein and completely degraded during the passage of the liver. Another explanation might be, that the blood-brain barrier is impermeable for the free 4*t*-sphingenine during the myelination period. Further experiments are in progress to study this phenomenon.

In these and previous experiments[1] the effective and rapid utilization of ethanolamine and its derivative choline and their respective phosphate esters originating from carbon 1 and 2 of 4*t*-sphingenine and sphinganine for phosphatidylethanolamine and phosphatidylcholine synthesis is very striking. Therefore an additional pathway for the formation of the ethanolamine moiety of phospholipids and sphingomyelin seems to be the degradation of the long-chain bases sphinganine, 4*t*-sphingenine and 4-D-hydroxysphinganine[6], indicating the intimate correlation of sphingosine base degradation and phospholipid synthesis. Ethanolamine and choline therefore are ultimately derived from serine. This pathway is summarized in Fig. 2.

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Experimental

Synthesis of [1-^{3}H]4t-sphingenine ([1-^{3}H]erythro-DL-sphingosine): Erythro-ethyl-2-amino-3-hydroxy-4*t*-octadecenoate hydrochloride was synthesized according to SHAPIRO et al.[12] starting with tetradecanal. The amino-ester was reduced in the following manner: 26 mg of $LiAlH_4$ were refluxed in 30 m*l* of dry ether for 10 min.

[12] D. SHAPIRO, K. H. SEGAL and H. M. FLOWERS, J. Amer. chem. Soc. **80**, 1194 [1958].

The suspension was cooled to 10°C and 229 mg (0.68 mmoles) of *erythro*-ethyl-2-amino-3-hydroxy-4*t*-octadecenoate hydrochloride, suspended in 10 ml of dry ether, were added. After stirring for 5 min at 10°C 25.2 mg of $LiAl^3H_4$ were added and additional 10 mg of $LiAlH_4$ after the reaction had proceeded at room temperature for 45 min. Subsequently the reaction mixture was refluxed for two hours. Finally the excess of $LiAlH_4$ was decomposed at 0°C with ethyl acetate and water. A concentrated solution of Na_2CO_3 was used for dissolving aluminiumhydroxide and the product was extracted with ether. The combined extracts were washed with water, dried over Na_2SO_4 and evaporated to dryness. Yield: 176 mg (0.59 mmoles), 86.8% of theory. The [1-^{3}H]4*t*-sphingenine was immediately transferred into the hydrochloride with HCl in dry ether. (m. p. 65—66°C, specific activity 1.28 μC/μmole). The product proved to be pure in thin-layer chromatographic analysis. Gas-liquid chromatography of the *N*-acetyl-di-*O*-trimethylsilyl derivative indicated, that only the *erythro*- and no *threo*-isomer was obtained.

Administration of the [1-^{3}H]erythro-DL-4t-sphingenine hydrochloride: Eight suckling rats, 13 days old, were treated with five subsequent intraperitoneal injections of 0.2 ml solutions of [1-^{3}H]*erythro*-DL-4*t*-sphingenine hydrochloride. Each dose contained 1 mg of [1-^{3}H]-*erythro*-DL-4*t*-sphingenine hydrochloride (specific activity 1.28 μC/μmole), 10 mg of albumin, 1.8 mg of NaCl and 0.5 mg of Triton WR 1339. The animals were injected on the 13th, 15th, 17th, 19th and 21th postnatal day. Seven rats were killed on the 26th day.

Extraction, separation and identification of the lipids: The brains and livers of 7 animals were removed, homogenized with an Ultraturrax and extracted by refluxing in 20 volumes of chloroform/methanol 2:1 for 15 min. This procedure was repeated twice. The extracts were filtered and evaporated to dryness under reduced pressure. The residue was dried over P_2O_5, dissolved in chloroform, filtered and an aliquot applied to a column of 60 g of silicic acid (2 cm ∅, 100—200 mesh, Mallinckrodt). The silicic acid had been previously washed with water and methanol several times and activated at 110°C for two hours. Chromatography was carried out as described before[2]. 10 ml fractions were collected automatically. Aliquots of 50 μl of each fraction were used for the measurement of radioactivity and the purity was checked by thin-layer chromatography. The compounds were identified by chromatography with authentic samples.

Enzymatic hydrolysis of phosphatidylethanolamine and phosphatidylcholine with Bac. cereus phospholipase C

Phospholipase C was obtained from a culture filtrate of *Bac. cereus*. The preparation of a crude enzyme extract was carried out according to HAVERKATE and VAN DEENEN[13]. To 100 ml of the clear filtrate 60 g of ammonium sulfate were added. The solution was allowed to stand for 12 h at 0°C then centrifuged at 20000 × *g* for 20 min (Sorvall RC 2) and the precipitate was dissolved in 6 ml of 0.1M Tris buffer (pH 7.2). The solution was cleared by centrifugation.

Hydrolysis with phospholipase C was achieved at room temperature according to the following procedure: 4 mg of phosphatidylethanolamine (2.48×10^5 dpm) and 30 mg of phosphatidylcholine respectively (4.4×10^5 dpm) in 1 ml of ether were stirred with 0.3 ml of the crude enzyme extract. After one hour the two phases were allowed to separate. The water soluble products phosphorylethanolamine and phosphorylcholine respectively were identified by thin-layer chromatography. The plates were scanned with a radiochromatogram scanner Packard Model 7201.

Thin-layer chromatography: Thin-layer chromatography was carried out on activated silicagel G plates (250 μ thickness). Depending on the products the following solvent systems were used:

[1-^{3}H]*erythro*-DL-4*t*-sphingenine: chloroform/methanol/2N NH_4OH 40:10:1[14].

Ceramide: chloroform/methanol 8:1.

Phospholipids: chloroform/methanol/water 65:25:4.

MN 300 cellulose plates (15 g of the powder homogenized in 90 ml of distilled water, cellulose MN 300, Macherey, Nagel & Co., Düren, Germany) were used for the chromatography of phosphorylethanolamine (solvent system: phenol (freshly distilled)/water 9:1) and phosphorylcholine (solvent system: butanol/glacial acetic acid/water 40:20:20).

Gas-liquid chromatography: For the separation of *threo*- and *erythro*-isomers of 4*t*-sphingenine and sphinganine by gas-liquid chromatography the *N*-acetyl-di-*O*-trimethylsilyl derivatives were prepared according to CARTER and GAVER[10]. The analyses were carried out on a 3.8% SE 30 on Chromosorb column (length: 1.8 m, temperature: 200°C) using a Packard gas chromatograph model 7820.

Radioactivity measurements: Radioactivity measurements were carried out in a liquid scintillation counter model 3214 Packard, La Grange, U.S.A., using the following scintillation liquids: 5 g of PPO (2,5-diphenyloxazole), 0.3 g of POPOP (1,4-bis-[4-methyl-5-phenyloxazolyl-(2)-benzene] in 1 l of toluene). BRAY's solution[15] was used for counting radioactive bands obtained from thin-layer plates.

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[13] F. HAVERKATE and L. L. M. VAN DEENEN, Biochim. biophysica Acta [Amsterdam] **106**, 78 [1965].

[14] K. SAMBASIVARAO and R. H. MCCLUER, J. Lipid. Res. **4**, 106 [1963].

[15] G. A. BRAY, Analyt. Biochem. [New York] **1**, 279 [1960].