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The Relationship between Phospholipid Biosynthesis, Sphingosine Degradation and Serine Metabolism

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Summary: The relationship between the biosynthesis of the phospholipids, the degradation of sphingosine and the metabolism of serine has been studied. [1-$^3H_2$]DL-*erythro*-sphingosine and [3-^{14}C]DL-serine were used as substrates. Long term experiments with young rats (14th–21st day after birth) and short term experiments with adult rats yielded the following similar results:
The total ^{3}H-radioactivity was localized in the ethanolamine and choline moieties of phosphatidylethanolamine and phosphatidylcholine. [^{14}C]Serine was incorporated, although in minute amounts, into the bases and the glycerol of the phospholipids. ^{3}H-labeled phosphorylethanolamine, free [$^3H_2$]ethanolamine, [^{14}C]serine and [^{14}C]phosphorylethanolamine were identified as water soluble compounds.
Even after the injection of large amounts of [3-^{14}C]DL-serine (100 μmoles) only a very small incorporation of ^{14}C into the liver lipids was observed. The considerable labeling of the glycerol moiety of the phospholipids, di- and triglycerides is remarkable. In addition to glycerol the bases of the phospholipids were labeled and to a minor extent the sphingosine bases of ceramide and sphingomyelin. Phosphorylethanolamine was the main radioactive compound in the aqueous extract of the liver and in the urine. It is also formed in high yield from [3-^{14}C]DL-serine after incubation with rat liver microsomes.
The results demonstrate that the bases of the phospholipids are predominantly derived from sphingosine when serine and the long-chain base are available for the biosynthesis of phosphatidylethanolamine and phosphatidylcholine.

Zusammenfassung: *Beziehungen zwischen der Biosynthese der Phospholipoide, dem Sphingosinabbau und dem Serinstoffwechsel.* Die Bedeutung des Sphingosinabbaus und des Serinstoffwechsels für die Biosynthese der Basen der Phospholipoide wurde vergleichend untersucht. Als Substrate dienten [1-$^3H_2$]DL-*erythro*-Sphingosin und [3-^{14}C]DL-Serin. Sowohl in Langzeitexperimenten (7 Tage) an 14–21 Tage alten Ratten als auch in Kurzzeitexperimenten mit erwachsenen Ratten (3 Std.) fanden wir folgende übereinstimmenden Ergebnisse:
Die gesamte Tritiumaktivität von Kephalin und Lecithin war nur im Äthanolamin bzw. im Cholin nachweisbar. Sphingomyelin war mit ^{3}H im Ceramid und im Cholin markiert. ^{14}C-Radioaktivität des Serins wurde, wenn auch in geringen Mengen, zu verschiedenen Anteilen in die Basen und in das Glycerin der Phospholipoide eingebaut. Als wasserlösliche Verbindungen wurden ^{3}H-markiertes Phosphoryläthanolamin und freies Äthanolamin sowie ^{14}C-Serin und ^{14}C-Phosphoryläthanolamin identifiziert.
Auch nach Injektion größerer Mengen [3-^{14}C]DL-Serin (100 μMol) wurde wiederum nur wenig ^{14}C-Aktivität des Serins in die Leberlipoide inkorporiert. Bemerkenswert war die hohe Markierung des Glycerins in Phospholipoiden, Di- und Triglyceriden. Die Hauptmenge der Radioaktivität der Phospholipoide trugen die Basen. Geringe Aktivitäten wurden in der Sphingosinbase der Sphingolipoide Ceramid und Sphingomyelin gefunden.

Enzyme:

Phospholipase C, phosphatidylcholine cholinephosphohydrolase (EC 3.1.4.3).

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Phosphoryläthanolamin war das Hauptprodukt unter den wasserlöslichen radioaktiven Verbindungen. Auch bei In-vitro-Inkubationen mit Rattenlebermikrosomen und [3-^{14}C]DL-Serin wurde Phosphoryläthanolamin in hoher Ausbeute (bis zu 50%) gebildet.

Die Ergebnisse zeigen deutlich, daß die Ausgangsprodukte für die Biosynthese der Basen der Phospholipoide bei gleichzeitigem Angebot von Sphingosin und Serin nahezu ausschließlich aus dem Sphingosinabbau stammen.

Recent investigations[1–4] in this laboratory have demonstrated that labeled sphingosine bases are degraded in the rat liver *via* the 1-phosphate derivative to a C_{16}-fragment, which has been identified as palmitaldehyde and to a C_2-unit, derived from carbon atom 1 and 2 of the long-chain base, which proved to be phosphorylethanolamine. These are incorporated rapidly and in high yield into the polar groups of the phospholipids[2,5].

Many studies have been reported concerning the formation of the polar groups of the phospholipids[6–14]. The best defined pathway for the *de novo* biosynthesis of phosphatidylethanolamine and phosphatidylcholine, developed by KENNEDY and WEISS[15], involves the transfer of phosphorylethanolamine and -choline from their CDP-derivatives to the D-2,3- diglyceride. Also the conversion of ethanolamine to phosphorylethanolamine[16] and the formation of phosphorylcholine in kinase reactions[17] and the stepwise methylation of phosphatidylethanolamine[18] are well known reactions. The CDP-derivatives are formed in reversible reactions catalyzed by the phosphorylethanolamine cytidyl transferase and phosphorylcholine cytidyl transferase respectively[15].

The conversion of serine into ethanolamine or into phosphorylethanolamine is an obscure reaction. STETTEN[6] demonstrated that serine is the common precursor of ethanolamine and choline. Further observations[11,12] indicate that the decarboxylation of serine may take place within the phospholipid molecule. However, at the present time the precise reactions and enzymes of the animal cell involved in the decarboxylation of serine are unknown. Only an exchange reaction between serine and the ethanolamine moiety of phosphatidylethanolamine has been described[10].

On the basis of our previous experiments we suggested a close relationship between the degradation of the sphingosine bases and phospholipid biosynthesis.

In this paper we want to report experiments concerning the utilization of sphingosine bases and of serine for the biosynthesis of phospholipids in the rat liver.

Results

In a long term experiment with four weanling rats the labeling of lipids after simultaneous injections of [1-$^3H_2$]DL-*erythro*-sphingosine hydrochloride and [3-^{14}C]DL-serine was investigated ($^3H/^{14}C$ ratio of specific activities 10.8). The labeled compounds were injected five times intraperitoneally at intervals of 36 h starting on the 14th day. On the 21th day the livers and the brains were pooled separately and the total lipids extracted. After the separation by silicic acid column chromatography

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Table 1. ^{3}H and ^{14}C uptake into the lipids of the liver and the brain of 4 weanling rats. Intraperitoneal injection of [1-$^{3}H_2$]DL-*erythro*-sphingosine hydrochloride (67.2 μmoles, 85.6 μC) and of [3-^{14}C]DL-serine (67.2 μmoles, 7.9 μC) ($^{3}H/^{14}C$ ratio = 10.8).

	Liver					Brain	
	$10^{-3} \cdot {}^{3}H$-Activity* [dpm]	[%]	$10^{-3} \cdot {}^{14}C$-Activity** [dpm]	[%]	$^{3}H/^{14}C$	$10^{-3} \cdot {}^{3}H$-Activity*** [dpm]	[%]
Ceramide	1879	10.3	1.21	6.6	1555	29.1	2.1
Phosphatidylethanolamine	7847	43.0	9.79	54.1	801	626	45.8
Phosphatidylcholine	6864	37.6	6.02	33.0	1141	673.45	49.2
Lyso-phosphatidylcholine						4.7	0.3
Sphingomyelin	1663	9.1	1.15	6.3	1452	35.6	2.6

* Total incorporation: 14.1%. ** Total incorporation: 0.18%. *** Total incorporation: 2%.

Table 2. ^{3}H and ^{14}C incorporation into the liver lipids of an adult rat. Intraportal injection of [1-$^{3}H_2$]DL-*erythro*-sphingosine hydrochloride (6.74 μmoles, 8.6 μC) and of [3-^{14}C]DL-serine (35 μmoles, 17.5 μC) ($^{3}H/^{14}C$ ratio = 0.49).

	$10^{-3} \cdot {}^{3}H$-Activity* [dpm]	[%]	$10^{-3} \cdot {}^{14}C$-Activity** [dpm]	[%]	$^{3}H/^{14}C$
Ceramide	428	11.6	1.36	1.3	314
Phosphatidylethanolamine	2056	56.8	31.56	30.6	65
Phosphatidylcholine	682	18.4	65.87	64.0	10.3
Lyso-phosphatidylcholine	21.7	0.6			
Sphingomyelin	464.7	12.6	4.1	4.1	113

* Total incorporation: 21.6%. ** Total incorporation: 0.37%.

the radioactivity of each lipid fraction was determined. Quantitative data on the separations of liver and brain lipids are given in Table 1.

By far the greatest amount of radioactivity incorporated into the total lipids of liver and brain was derived from sphingosine. The liver lipids contained 14.1% and the brain lipids 2% of the injected ^{3}H-radioactivity. Less than 0.3% of [3-^{14}C]serine was found in the total lipids of the liver and the brain. The ^{3}H-distribution in the lipid classes clearly shows, that the maximum incorporation was into the phospholipids of the liver (>80%) and of the brain (>90%). The sphingolipids of the liver are significantly more highly labeled than those of the brain. The ^{14}C-distribution of the lipids in the liver is similar to that of ^{3}H. In brain the ^{14}C-radioactivities of the isolated lipid fractions were too low (0.09%) to be determined exactly. The considerable increase in the $^{3}H/^{14}C$-ratios starting from 10.8 up to about 1000 for the phospholipids and to approx. 1500 for the sphingolipids demonstrates strikingly the importance of sphingosine for the biosynthesis of these substances.

The data of a short term experiment with an adult rat support the results described above. In this investigation a $^{3}H/^{14}C$ ratio of 0.49 was taken, the molar ratio of sphingosine/serine being 0.19. Both compounds were administered into the portal vein of an adult rat. This technique provides the most effective incorporation of labeled material into the liver lipids. After 3 h the total liver and brain lipids were extracted and separated by column chromatography. Table 2 shows the distribution of the isotopes in the lipid classes. Similar to the long term experiment sphingosine represents the most important source for the radioactivity supply for lipid synthesis. 21.1% of the total ^{3}H radioactivity was present in the liver and 0.3% in the brain. Except for the low incorporation into phosphatidylcholine the distribution of ^{3}H-labeled lipids is similar to that of the long term experiment.

Again more than 75% of the ^{3}H-radioactivity was

incorporated into the phospholipids, the rest mainly as sphingosine into the sphingolipids. Free labeled long-chain bases could not be detected.

The ^{14}C-radioactivity derived from serine in the lipids did not exceed 0.4%. The distribution pattern of the identified lipids resembled closely the results of the long term experiment. The small uptake of sphingosine into the sphingolipids is very striking. Here too, the ratios of $^{3}H/^{14}C$ increased from 0.49 up to 10—60 for the phospholipids and 100—300 for the sphingolipids respectively.

Further information on the localisation of the label in the lipid molecules was obtained by the enzymic hydrolysis of the phospholipids with phospholipase C. This treatment yielded phosphorylserine, phosphoryl ethanolamine and phosphorylcholine as water soluble products. They were separated and identified by paper electrophoresis. The ether soluble products were analysed by radio-thin-layer chromatography. The results are summarized in Table 3.

Table 3. Intramolecular distribution of ^{3}H- and ^{14}C-radioactivity in rat liver phospholipids, determined by enzymic hydrolysis with phospholipase C.

		Phosphate esters of bases	Diglyceride
		Radioactivity [%]	
Phosphatidyl-ethanolamine	(^{3}H)	96	4
	(^{14}C)	53.5	46.4
Phosphatidyl-choline	(^{3}H)	98	2
	(^{14}C)	62.4	37.6
Sphingomyelin	(^{3}H)	22.5	77.5 (ceramide)

The total ^{3}H-radioactivity of phosphatidylethanolamine and phosphatidylcholine was localized in the phosphorylethanolamine and phosphorylcholine moiety, respectively. Ceramide was the main radioactive hydrolysis product of sphingomyelin. The distribution of ^{14}C in the phospholipid molecules was similar to that described in the [3-^{14}C]serine experiment and will be reported jointly below. The total uptake of both isotopes into the brain lipids did not exceed 0.2% and therefore the isotope distribution was not further investigated.

The loss of ^{3}H-radioactivity in the urine was 2.7%, 90% of which was phosphorylethanolamine and -choline and the rest was free ethanolamine and choline.

In another experiment [3-^{14}C]serine was administered in higher amounts (100 μmoles) in order to obtain more detailed information on its metabolic fate. The results indicate that within a 3 h experiment 14% of the radioactivity appeared as $^{14}CO_2$, 8% in the urine and 2% in the aqueous extract of the liver, whereas only 0.6% of the injected radioactivity was incorporated into the liver lipids.

Fig. 1 and Table 4 show the distribution of ^{14}C in the total lipids after chromatographic separation. More than 75% of the radioactivity was found in the phospholipids. The high uptake of ^{14}C into the triglycerides should be mentioned. The transfer of serine into the sphingolipids however did not exceed 4% of the total radioactivity in the lipid extract.

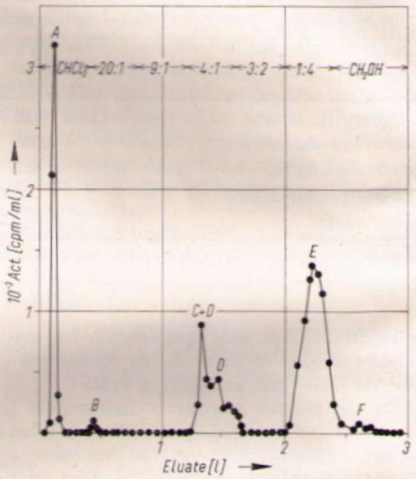

Fig. 1. Silicic acid chromatography of the total lipid mixture from rat liver after the administration of 100 μmoles (50 μC) of [3-^{14}C]DL-serine.

Fractions: A: triglycerides; B: ceramide; C: phosphatidylethanolamine; D: phosphatidylserine; E: phosphatidylcholine; F: sphingomyelin.

The investigation of the intramolecular localisation of the label (see Table 4) was carried out by two different methods of hydrolysis. After mild alkaline hydrolysis of the triglycerides the total radioactivity was found in glycerol identified by radio-thin-layer chromatography.

Table 4. Incorporation of ^{14}C into liver lipids after the intraportal injection of [3-^{14}C]DL-serine (100 μmoles, 50 μC) showing the specific activities and the intramolecular distribution of radioactivity in the purified lipids.

	10^{-3} · Activity [dpm]	Radioactivity* [%]	Specific activity [dpm/μmole]	Radioactivity [%] glycerol	polar groups
Triglycerides	111.65	16.6	1010	100	
Diglycerides	6.45	0.9			
Ceramide	7.45	1.2			
Phosphatidylserine	74.2	11	4605	6	94
Phosphatidylethanolamine	102.25	15	681	47.8	52.2
Phosphatidylcholine	355.55	52.8	1162	26.4	73.6
Sphingomyelin	16.9	2.5	844	59.5 (ceramide)	40.5

* 0.6% of the injected radioactivity was incorporated in the liver lipids.

Enzymic hydrolysis of the phospholipids with phospholipase C yielded labeled diglycerides and water soluble compounds. Further hydrolysis of the diglycerides by mild alkaline hydrolysis showed only glycerol to be radioactive and not the fatty acids. The water soluble products phosphorylserine, phosphorylethanolamine and phosphorylcholine were identified by paper electrophoresis and by scanning. Enzymic hydrolysis of sphingomyelin released labeled ceramide and the less radioactive phosphorylcholine. The quantitative data of these procedures are summarized in Table 4.

The fate of [3-^{14}C]DL-serine not incorporated into lipids was investigated by paper electrophoretic analysis of the aqueous phase of the liver and of the urine collected during the 3 h period of the experiment. The data are given in Table 5. Attention is drawn to the high amount of the administered radioactivity present in the aqueous extract of the liver and in the urine and to the predominant occurrence of ethanolamine and choline as phosphate esters.

Table 5. Distribution of ^{14}C-labeled compounds in the aqueous phase of the liver and in the urine after the injection of [3-^{14}C]DL-serine (100 μmoles, 50 μC).

	Radioactivity [%] Aqueous extract of liver*	Urine**
Serine	28.2	48.6
Phosphorylethanolamine	54.0	36.5
Phosphorylcholine	17.3	14.4
Ethanolamine + choline	0.5	0.5

* Total incorporation: 2% of the injected radioactivity.
** Total incorporation: 8% of the injected radioactivity

In preliminary experiments rat liver microsomes transformed [3-^{14}C]DL-serine mainly into phosphorylethanolamine (Fig. 2), identified by electrophoresis and as its dinitrophenyl derivative by radio-thin-layer chromatography.

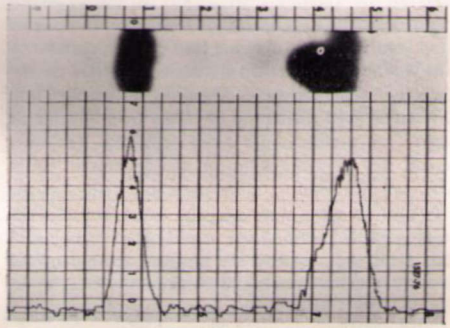

Fig. 2. Distribution of radioactivity after paper electrophoresis of the aqueous phase of rat liver microsomes after incubation with 10 μmoles (5 μC) of [3-^{14}C]DL-serine.
A: phosphorylethanolamine; B: serine. (Staining by ninhydrin spray).

Discussion

The results of our experiments indicate a close relationship between the degradation of sphingosine bases and the biosynthesis of phospholipids. After the simultaneous parenteral administration of

[1-$^3H_2$]DL-*erythro*-sphingosine and [3-^{14}C]DL-serine, the ^{3}H-incorporation into the phospholipids exceeds the incorporation of ^{14}C by a factor of 50 in the adult and by a factor of 80 in the young rats. Enzymic hydrolysis of phosphatidylethanolamine, phosphatidylcholine and sphingomyelin with phospholipase C showed that all tritium activity was associated with phosphorylethanolamine and phosphorylcholine. This result is in full agreement with our previous observations[2,5]. The radioactive carbon derived from serine could be detected in the phosphorylethanolamine and in the phosphorylcholine moiety as well as in the diglyceride moiety of the phospholipids.

The utilization of ^{3}H-labeled sphingosine for the biosynthesis of the phospholipids is much more favoured than that of serine. This is obvious from the approximate 100fold increase of the $^3H/^{14}C$ ratio in the phospholipids over the ratio of the isotopes of the injected compounds. In the present experiments sphingosine is incorporated into sphingolipids without any breakdown of the molecule. During the interval between the 10th and 20th day after birth, brain sphingolipids of young rats have a very low turnover rate[19]. This might explain the different distribution pattern of radioactive lipids in liver and brain of these animals. The low incorporation of serine into the lipids led us to the assumption that an extensive dilution of the injected serine by the endogenous serine pool and by the *de novo* formation of serine from glycine or other precursors might have occurred. To overcome this possible dilution and to obtain more detailed information on the metabolic fate of serine 100 μmoles were injected. The large amounts of radioactivity found in the respiratory air (14%), in the urine (8%) and in the aqueous extract of the liver (2%) compared to the small incorporation into lipids (0.6%) point to the minor role of serine as a direct precursor in lipid biosynthesis.

Four pathways for the utilization of serine in lipid biosynthesis could be envisaged. The conversion of [3-^{14}C]DL-serine into the glycerol moiety of the triglycerides is very striking and so far no experimental data have been reported concerning this transformation except a short report of ANSELL and SPANNER[20] dealing with the incorporation of serine into the glycerol moiety of phospholipids. The intramolecular ratios of the radioactivities of the polar groups and of the diglyceride groups of the different phospholipids make the assumption unlikely that phosphatidylethanolamine and phosphatidylcholine are predominantly formed by the conversion of phosphatidylserine[12,20].

The high specific radioactivity of the triglycerides suggest different sources of the D-2,3-diglycerides for the biosynthesis of both the triglycerides and the phospholipids, especially if one considers that the major amount of radioactivity in the phospholipids derived from serine was localized in their ethanolamine or choline moiety. Similar results were obtained by MARINETTI[21]. He incubated rat liver homogenates with [^{14}C]glycerol and observed after esterification with different fatty acids different conversion rates of the newly synthesized diglycerides into triglycerides and phospholipids.

Another important pathway is the incorporation of the total serine molecule into phosphatidylserine, its conversion into the ethanolamine and choline moiety of phosphatidylethanolamine and phosphatidylcholine and the formation of the methyl groups of choline from the C-3-atom of serine.

A further possibility is the condensation of serine with acyl-CoA to 3-oxo-dihydrosphingosine[22,23]. Taking all these pathways into account we found in a typical experiment the following utilization of [3-^{14}C]DL-serine radioactivity: 39.4% as glycerol, 58% as polar groups of the phospholipids and 2.4% as sphingosine bases of the sphingolipids.

The large amounts of phosphorylethanolamine derived from serine, found in the aqueous extract of the liver as well as in the urine, obviously suggest that this compound is a direct degradation product of serine. Also in studies *in vitro* with rat liver microsomes up to 50% of the total serine is converted into phosphorylethanolamine. However at the moment no mechanism for the direct decarboxylation of serine to ethanolamine is known nor can be derived from these experiments. The exchange reactions between serine and phosphatidylethanolamine to form phosphatidylserine and free ethanolamine, described by BORKENHAGEN and

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KENNEDY[13], cannot be considered to produce sufficient amounts of ethanolamine in the mammalian cell.

Evidence for a defined reaction sequence for the formation of phosphorylethanolamine has been obtained on the basis of our experiments[2–5] on the degradation of sphingosine bases. By this mechanism it is possible to supply the *de novo* synthesis of phosphatidylethanolamine with the respective CDP-derivatives required for its formation[15].

Under our experimental conditions phosphorylethanolamine is formed in sufficient amounts for the biosynthesis of the phospholipids from the two precursors serine and sphingosine. Since the pathway from phosphorylethanolamine *via* the CDP-intermediate is well established[15] the question arises by which mechanism the cell prefers phosphorylethanolamine derived from sphingosine to that formed by the degradation of serine. This phenomenon can be explained in the following ways:

1. Sphingosine bases are metabolized in the cell by two pathways. They are either used intact for the biosynthesis of the sphingolipids or they are degraded. Because of the low incorporation rates of the sphingolipids[19] only small amounts of sphingosine bases are used. Every excess of long chain bases immediately after phosphorylation will be degraded and provides free phosphorylethanolamine. Serine, however, is rapidly metabolized by many pathways according to the experiments reported here and to observations described by NEMER[24], who worked with rat liver slices and [3-^{14}C]DL-serine. These authors found a negligible utilization of serine for the biosynthesis of lipids (2%) compared to the loss into the extracellular space (37%), C_1-metabolism (47%), gluconeogenesis (7%) and protein biosynthesis (5%).

2. Sphingosine, once introduced into the cell, will be located in the membranes due to its hydrophobic nature. The sphingosine-degrading enzymes are bound to the membranes of the endoplasmic reticulum and the mitochondria, which are also the site of phospholipid biosynthesis. The close spatial relationship between these two systems therefore favours an easy and rapid transfer of phosphorylethanolamine to the sites of the biosynthesis of the phospholipids and the extensive and preferred utilization of the phosphorylethanolamine liberated at these sites.

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Experimental

[1-^{3}H$_2$]DL-*erythro*-Sphingosine hydrochloride was synthesized as previously described[5,25] (specific radioactivity 1.28 mC/mmole). The compound proved to be pure in thin-layer chromatography. The *erythro*-configuration was established by gas-liquid chromatography of the *N*-acetyl-di-*O*-trimethyl silyl derivative[26]. [3-^{14}C]-DL-Serine (specific activity 53 mC/mmole, diluted to 0.5 mC/mmole) was purchased from the Radiochemical Centre, Amersham, Great Britain.

In vivo procedures

Simultaneous injections of [1-^{3}H$_2$]*erythro*-DL-sphingosine hydrochloride and [3-^{14}C]DL-serine: Eight weanling rats, 14 days old were treated with five subsequent intraperitoneal injections (0.2 m*l*) at intervals of 36 h. 0.2 m*l* of the injection solution contained the following components: 3.36 μmoles (4.2 μC) of [1-^{3}H$_2$]DL-*erythro*-sphingosine hydrochloride, 3.36 μmoles (0.38 μC) of [3-^{14}C]DL-serine, 1.8 mg of NaCl, 10 mg of albumin and 0.5 mg of Triton WR 1339. Four rats survived and were killed on the 21th day after birth. The livers (8.35 g) and the brains (4.33 g) were immediately removed, pooled and extracted as described under extraction procedures.

Also an adult rat was injected *via* the portal vein after abdominal incision with 1 m*l* of the following solution: 6.7 μmoles (8.6 μC) of [1-^{3}H$_2$]DL-*erythro*-sphingosine hydrochloride, 35 μmoles (17.5 μC) of [3-^{14}C]DL-serine, 9 mg of NaCl, 1 mg of Triton WR 1339 and 20 mg of albumin. The injection was carried out under ether anaesthesia.

Similarly 1 m*l* of a solution containing 100 μmoles (50 μC) of [3-^{14}C]DL-serine and 9 mg of NaCl was injected into the portal vein of two adult rats. After 3 h the livers were removed and the lipids extracted.

In vitro incubations

Rat liver microsomes were prepared in the usual manner[27]. Protein was determined by the biuret method. 10 μmoles (5 μC) of [3-^{14}C]DL-serine were incubated for 2 h at 37°C in a total volume of 2 m*l* 0.25M sucrose and 0.1M phosphate buffer, pH 7.4, containing 23 mg of protein, 10 μmoles of ATP, 1 μmole of CoA, 1 μmole of pyridoxal phosphate, 5 μmoles of $MgCl_2$, 5 μmoles of thioglycol, 5 μmoles of CMP.

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Extraction, separation and identification of the labeled products

Livers and brains were homogenized with an Ultra-Turrax. The homogenates and the lyophilized incubation mixtures were extracted twice by refluxing in 20 volumes of chloroform/methanol 2:1 (v/v). The pooled extracts were washed three times with half a volume of water. In addition the delipidized residue was washed three times with water. The aqueous extracts were pooled, acidified with HCl and concentrated under reduced pressure. The water-soluble labeled compounds were separated and identified by paper electrophoresis and paper chromatography. The water-washed lipid extracts were concentrated in a nitrogen atmosphere under reduced pressure at 37°C. The residue was dried over P_2O_5, dissolved in chloroform and applied to a column of activated silicic acid (Mallinckrodt 100 to 200 mesh) (1 g silicic acid/5 mg of lipid). The elution with chloroform and increasing amounts of methanol was carried out as described before[1]. 10 ml fractions were collected automatically. Aliquots of the fractions were used for the measurement of radioactivity. The labeled compounds were identified by thin-layer chromatography with authentic samples.

Thin-layer chromatography

The thin-layer chromatography was carried out on activated silica gel G plates (250 μ thickness). Depending on the compounds the following solvent systems were used:

long chain bases: chloroform/methanol/NH_4OH (2N) 40:10:1[28];

neutral lipids: petroleum ether (30—60°C)/ether/acetic acid 90:10:1 or 70:30:2;

ceramide: chloroform/methanol 15:1;

phospholipids: chloroform/methanol/water 65:25:4 and chloroform/methanol/acetic acid/water 25:15:4:2[29];

glycerol: chloroform/acetone/NH_4OH (5N) 10:80:10[30];

dinitrophenyl-derivatives of serine and phosphorylethanolamine on silica gel H plates: butanol/acetic acid/water 80:20:20.

The 2,4-dinitrophenyl derivatives of serine and phosphorylethanolamine were synthesized according to LEVY[31]. Dinitrophenyl-serine was separated from the water-soluble dinitrophenyl-phosphorylethanolamine by extracting the acidified reaction mixture with ether. The separated labeled dinitrophenyl-derivatives were identified by chromatography on silica gel H plates together with inactive authentic samples and scanned with a radiochromatogram scanner, Packard, Model 7201.

Paper chromatography

Phosphorylcholine was separated from phosphorylethanolamine by ascending paper chromatography (Schleicher & Schüll 2045b) with the solvent system phenol (freshly distilled)/water/formic acid 90:10:1[12] (R_F values for phosphorylethanolamine 0.4 and for phosphoryl choline 0.85).

Paper electrophoresis

25×5 cm paper strips (Macherey & Nagel MN 214) were used in a Gelman electrophoresis chamber with the buffer system: 0.4M formic acid and 0.53M acetic acid (pH 2)[32]. Under these conditions ethanolamine and choline were separated together from the slowly running labeled compounds, phosphorylethanolamine, phosphorylcholine and serine in a 1.5 h run (12 V/cm). Serine and phosphorylserine could be separated in the same buffer system from phosphorylethanolamine and phosphorylcholine (running together) in a 4 h electrophoresis at 12 V/cm. The R_F values relative to serine (1.0) were: phosphorylethanolamine and phosphorylcholine (0.5) and phosphorylserine (0.25—0.3).

Enzymic hydrolysis of phospholipids and sphingomyelin with phospholipase C

Phospholipase C (EC 3.1.4.3) was obtained from a culture filtrate of *Bacillus cereus*. The preparation of a crude enzyme extract was carried out according to HAVERKATE and VAN DEENEN[33]. Lipid fractions (3 to 5 mg), dissolved in 2 ml of ether, were hydrolyzed at room temperature with 1 ml of the crude enzyme extract. The two-phase system was stirred vigorously over a period of 4—6 h. The hydrolysis was followed by thin-layer chromatography (solvent system: chloroform/methanol/water 65:25:4). The ether layer contained the diglycerides or ceramides respectively and the aqueous phase the phosphate esters of the bases. Both phases were concentrated under vacuum and the diglycerides identified by thin-layer chromatography and the phosphorylated water-soluble hydrolysis products by paper electrophoresis or paper chromatography.

Specific radioactivities

The specific radioactivities of the phospholipids were determined by a modified BARTLETT procedure[34,35] and

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that of the triglycerides, purified by thin-layer chromatography, by the determination of ester groups according to SNYDER and STEPHENS[36]; the radioactivities were measured with BRAY's[37] solution as scintillator; the counting efficiency was determined by the internal standard method.

Radioactivity measurements

For radioactivity measurements a liquid scintillation counter model 3214 Packard, La Grange, USA, was used and the following scintillation liquids: 5 g of PPO (2,5-diphenyloxazole), 0.3 g of POPOP (1,4-bis[4-methyl-5-phenyl-2-oxazolyl-benzene]) in 1 *l* of toluene. BRAY's solution was used for counting radioactive bands obtained from thin-layer plates as well as for water containing solutions. Respiratory $^{14}CO_2$ was collected and measured by a procedure previously described[1] with a scintillator[38] containing ethanolamine. The thin-layer plates and paper strips of the electrophoresis and paper chromatography were scanned in a Packard chromatogram scanner, model 7201.

Spraying reagents for chromatography

For the identification of compounds in thin-layer chromatography the charring technique (5% CrO_3/50% H_2SO_4), or staining with iodine vapour were used. Compounds containing amino groups were detected with ninhydrin reagent, phosphate groups with a reagent according to DITTMER and LESTER[39]. Glycerol was identified by a specific identification procedure described by CIFONELLI and SMITH[40]. Mild alkaline hydrolysis of the triglycerides and diglycerides was carried out according to DAWSON[41].

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