ON THE BIOSYNTHESIS OF CEREBROSIDES: NONENZYMATIC *N*-ACYLATION OF PSYCHOSINE BY STEAROYL COENZYME A

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1. Introduction

In vitro and *in vivo* experiments have indicated two pathways for cerebroside biosynthesis (for references, see [1]). Psychosine and ceramide, respectively, were proposed as intermediates in these pathways. Originally, the *in vitro* conversions of UDP [^{14}C]galactose [2, 3] or [1-^{14}C]stearoyl CoA [4] to cerebrosides in the presence of unlabeled lipid substrates were measured. Both pathways have also recently been examined with doubly deuterium labeled ceramides [1, 5] and dihydropsychosine [6]. Label originating in these substrates could be detected in the cerebrosides isolated after incubations similar to those described in [2–4]. It was furthermore shown that the substrates were not hydrolyzed and recombined during the transformations to cerebrosides. The present report provides evidence that the conversion of psychosine to cerebrosides does not require the participation of enzyme. Other primary amines also reacted nonenzymatically with stearoyl CoA under similar conditions, to form amides.

* Abbreviations and trivial names used in the text: CoA, coenzyme A; GLC, gas-liquid chromatography; MS, mass spectrometry; psychosine, 1-*O*-(β-D-galactopyranosyl) D-erythro-1, 3-dihydroxy-2-amino-4-*trans*-octadecene; sphingosine, D-erythro-1, 3-dihydroxy-2-amino-4-*trans*-octadecene; TGCU, triglyceride carbon units [18]; TLC, thin layer chromatography; TMSi, trimethylsilyl; UDPGal, uridinediphosphogalactose, UDPGlc, uridinediphosphoglucose; UV, ultraviolet.

2. Experimental procedure

2.1. *Chemicals*

Psychosine was prepared from brain cerebrosides (grade II, Sigma Chemical Company, St. Louis, Mo.) [7] and purified by TLC* [6]. A mass spectrum of the penta-*O*-TMSi derivative (C-value 32.3 on OV-1) indicated a molecular weight of 821 and was quite similar in appearance to that of the saturated analogue [6]. Sphingosine was a preparation from beef lung ceramide [8] and 1-n-dodecylamine was purchased from Kebo AB, Stockholm, Sweden. The amines were converted to sulfate salts and recrystallized as such [7]. Stearoyl CoA was prepared from [1-^{14}C]stearic acid (New England Nuclear Corp.; 1 μCi/μmole) using a mixed anhydride procedure [9]. It gave a single phosphorous positive spot on paper chromatography (H_2O–isopropanol–pyridine, 2:1:1 v/v/v; R_F value 0.7). The product had an adenine: thiol ester:organic phosphorous ratio of 1.00:0.95: 3.04. This was determined by absorbance at 260 nm [10], hydroxamic acid assay [10] and phosphorous determination [11]. The ratio of absorbance at 260 nm and 232 nm was 1.78 (cf. [10]). Treatment with 2 N NaOH (20°, 30 min) quantitatively released the radioactivity as stearic acid (identification: TLC and GLC of the methyl ester). Stearhydroxamic acid was prepared from ethyl stearate [12] and used as reference for the colorimetric determinations. Its purity was established by TLC (toluene–methanol, 8:2 v/v) and GLC [13]. *N*-dodecyl octadecanamide was prepared with a carbodiimide procedure [8]. The infrared spectrum had bands at 3450, 2930, 2860, 1660, 1518, 1468 and 680 cm^{-1} and the mass spectrum

Table 1
Silicic acid chromatography of lipid extracts obtained after incubations of psychosine and [1-^{14}C] stearoyl CoA.

Solvent	(a) Brain microsomes (dpm)	(b) Boiled microsomes (dpm)	(c) Without microsomes (dpm)
Chloroform	1,033,000	399,000	413,000
Acetone–methanol, 9:1	62,000	53,000	41,000
Methanol	182,000	29,000	37,000

The incubation mixture contained: 7.2 μmoles of psychosine sulfate, 0.57 μmole (1,260,000 dpm) [1-^{14}C] stearoyl CoA, ATP, $MgCl_2$, Tween 20, potassium phosphate buffer and fresh or heat inactivated (100°, 30 min) rat brain microsomes [4], as described in sect. 2.3. No microsomes were added in experiment (c). After 3 hr at 37° (N_2-atmosphere), $CHCl_3$–CH_3OH, 2:1 v/v was added. The washed lower cm^{-1} phase was concentrated and applied to the column [14].

ions at m/e 451 (M), M-29-n·14 (n=0–13)†, m/e 227 (McLafferty rearrangement ion and base peak), 212 and M-265-n·14 (n=0–5). *N*(stearoyl) sphingosine was prepared as described before [8].

2.2. *Analytical methods*

Silicic acid chromatography of cerebrosides [14] and TLC on borate impregnated plates [15] were performed as described before. The methods for radioactivity determinations, GLC and GLC–MS have also been described [1].

2.3. *Incubations*

The standard incubation mixture contained in 7.8 ml: 7.2 μmoles of amine sulfate, 0.57 μmole of stearoyl CoA, 16 μmoles of $MgCl_2$, 16 μmoles of ATP, 1 mmole of potassium phosphate buffer at pH 7.8 and 8 mg of Tween 20. Brain microsomes, when added, were prepared from 14 day old male Sprague Dawley rats [4]. 100 mg of protein (biuret) were used. The solvent of the incubation mixture was water for psychosine and 30% and 27% tetrahydrofuran in water, v/v, for sphingosine and dodecylamine, respectively. The incubations (37°, 3 hr in N_2-atmosphere) were stopped by addition of $CHCl_3$–CH_3OH, 2:1 v/v. The washed lower phase [1] was fractionated by silicic acid chromatography (eluants: acetone–methanol, 9:1 v/v for cerebrosides [14], ethyl acetate–benzene, 4:6 v/v, for ceramides and the same solvents mixed 5:95 v/v for *N*-dodecyloctadecanamide) followed by TLC (solvent systems: $CHCl_3$–CH_3OH–H_2O, 144:25:2.8 v/v/v, $CHCl_3$–CH_3OH 95:5 v/v and $CHCl_3$, respectively).

† Integral numbers only.

3. Results

3.1. *N-acylation of psychosine*

Table 1 shows the distribution of radioactivity in the silicic acid column chromatography fractions after incubations of psychosine and [1-^{14}C] stearoyl CoA with microsomes (a), heated microsomes (b) and without microsomes (c). About 70% of the radioactivity in each $CHCl_3$ eluate was free stearic acid as judged by TLC before and after methylation and by radio-GLC of the methyl ester. The extent of hydrolysis was 3 times higher with microsomes than with boiled microsomes or without microsomes. The main TLC component in the methanol eluate from experiment (a) (about 75% of the radioactivity) had an R_F value of 0.3 in $CHCl_3$–CH_3OH–H_2O 130:50:8 v/v/v, and was susceptible to mild alkaline methanolysis [8]. It was not further characterized. TLC of the acetone–methanol eluate from experiment (a) showed a product which cochromatographed with brain cerebrosides. On borate TLC [15] it behaved as galactosyl ceramide containing non-hydroxy acids. HIO_4–$NaBH_4$–HCl degradation and TLC separations [16] revealed that the label was in a saturated non-hydroxy acid ceramide. It has been shown before that labelled long chain base and hexose in the form of dihydropsychosine were converted to cerebrosides in the same system [6]. Heating the microsomes did not lower the amount of radioactivity in the cerebroside region and when protein was excluded, (c), there was a 6-fold increase in this product (table 2). The product formed in the absence of enzyme also cochromatographed with non-hydroxy acid galactosyl ceramides on borate

Table 2
Effect of heat denaturation of microsomes and omission of microsomes on the formation of cerebroside from psychosine and [1-^{14}C] stearoyl CoA.

Enzyme	Cerebroside formed (dpm)
Brain microsomes (a)	3,100
Boiled microsomes (b)	2,900
Without microsomes (c)	17,400

The acetone–methanol, 9:1, v/v, eluates of table 1 were concentrated and fractionated by TLC ($CHCl_3$–CH_3OH–H_2O, 144:25:2.8, v/v/v). The cerebroside fraction was detected and eluted from the plates as described before [1].

TLC. The latter had been added as carrier to the lipid extract. Radio-GLC showed a single radioactivity peak, with the same retention time as carrier cerebrosides** containing C_{18} acids (48.6 TGCU). The final proof for a cerebroside structure was provided by GLC–MS analysis of the product isolated without addition of carrier lipid. The chromatogram showed two components. The first one was not a cerebroside but the mass spectrum of the second component (48.6 TGCU) was in complete agreement with the structure penta-*O*-TMSi O^1-galactosyl *N*(stearoyl) sphingosine [17]. Table 3 illustrates some requirements for nonenzymatic cerebroside formation.

3.2. *N-acylation of sphingosine and 1-dodecylamine*

Sphingosine sulfate and 1-dodecylamine sulfate were incubated similarly to psychosine, except that the mixture contained tetrahydrofuran. Beef lung ceramide and *N*-dodecyloctadecanamide were added as carriers to the lipid extracts. These were subsequently fractionated by silicic acid chromatography and TLC. The latter technique revealed formation of labeled products with the same R_F values as the carrier lipids. The products were eluted and analyzed by radio-GLC. Both chromatograms showed two peaks of radioactivity. One emerged shortly after the solvent front, in both cases, whereas the second components (20% and 40% of the radioactivity, respectively) cochromatographed with *N*(stearoyl) sphingosine** and *N*-dodecyloctadecanamide (39.4 and 32.4 TGCU on OV-1). Finally, the products isolated after incubations without additions of carrier lipids were analyzed by GLC–MS. The chromatograms showed components with the expected retention times. Their mass spectra were indistinguishable from those of synthetic *N*(stearoyl) sphingosine** [18] and *N*-dodecyloctadecanamide (cf. above, sect. 2.1). Table 4 shows the amounts of *N*-acylation products formed from the three amines used here.

4. Discussion

Psychosine sulfate and [1-^{14}C] stearoyl CoA were incubated with brain microsomes under the conditions originally used for *in vitro* demonstration of cerebroside synthesis from psychosine [4]. This resulted in 0.24% conversion of stearoyl CoA to O^1-galactosyl *N*(stearoyl) sphingosine. 60% of the thiol ester was

Table 3
Some requirements for cerebroside formation from psychosine sulfate and [1-^{14}C] stearoyl CoA in the absence of microsomes.

Omissions	Additions	Cerebroside formed (dpm)
None	None	18,200
ATP	None	11,100
ATP + $MgCl_2$	None	12,800
ATP + $MgCl_2$ + K-PO_4	None	2,900
[1-^{14}C] stearoyl CoA	Na-[1-^{14}C] stearate	900

The complete system was that described in sect. 2.3. Cerebroside formation was assayed as described in the legends to tables 1 and 2. The specific activity of the sodium stearate was 1 μCi/μmole, and 0.57 μmoles were added.

**TMSi derivative.

Table 4
Effect of amine structure on the nonenzymatic *N*-acylation by [1-^{14}C]stearoyl CoA.

Amine	N-acylation product formed (dpm)
Psychosine	17,500
Sphingosine	15,200
1-n-Dodecylamine	12,500

Similar procedures to those described in the legends to tables 1 and 2 were used (see also sect. 2.3). The radioactivity eluted from the TLC plates after incubations with sphingosine and dodecylamine (but not psychosine) contained two radioactive components as judged by radio-GLC. The values shown in the table have been corrected for this.

hydrolyzed and 20% was converted to polar lipids during this incubation. With heat inactivated microsomes (100°, 30 min) thiol ester hydrolysis and polar lipid formation were reduced to 20% and 2%, respectively. The conversion to cerebroside was unaffected (0.23%). In the absence of microsomes it increased to 1.4%. The increase was probably due to protein binding of stearoyl CoA in the presence of microsomes [19]. Sodium stearate could not replace stearoyl CoA as cerebroside precursor. This demonstrates that activation of the fatty acid is necessary for the reaction. Non-enzymatic reactions between thiol esters and amines under physiological conditions of temperatures and pH have been reported [20, 21]. The structure of the thiol ester and the pK of the amine strikingly influenced the yield of amide. Thus, $CH_3CO{-}S{-}CH_2$-COOH and aniline reacted quantitatively to give acetanilide but $CH_3CO{-}S{-}CH_2CH_2NHCOCH_3$ gave only a few percent conversion. The latter compound structurally resembles the terminal *S*-acyl pantethein part of stearoyl CoA.

Omission of the potassium phosphate buffer (table 3) led to 75% reduction of cerebroside formation from psychosine. This may have been caused by decreased concentration of free amine secondary to an increase in hydroxonium ion concentration. The nonenzymatic reactions mentioned above [20, 21] were strongly pH-dependent, and an S_N2 mechanism with the free amine as a nucleophilic reagent was suggested. Two other examples of nonenzymatic acyl-transfer from CoA esters under mild conditions are well known: (i) the reaction with NH_2OH to give hydroxamic acid and CoA and (ii) the thiol ester interchange reaction [10].

In summary, the present results indicate that the psychosine pathway for cerebroside biosynthesis does not have any biological significance. It is unlikely that sufficient nonenzymatic synthesis via this pathway should occur *in vivo*. Several reports have described transformations of 2-hydroxy acid ceramides [2, 22, 1] and nonhydroxy acid ceramides [23, 3, 5] to the corresponding galactosyl and glucosyl ceramides. These reactions did not take place in the absence of active enzyme. It seems likely, therefore, that there is only one pathway for cerebroside synthesis *in vivo*, and that this pathway involves ceramide as an intermediate.

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