ENZYMIC SYNTHESIS OF CEREBROSIDE FROM GLYCOSYLSPHINGOSINE AND STEAROYL-CoA BY AN EMBRYONIC CHICKEN BRAIN PREPARATION

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Received November 14, 1973

SUMMARY:Glucosylceramide and galactosylceramide were enzymically synthesized by incubating an embryonic chicken brain microsomal fraction with stearoyl-CoA and,respectivelly,glucosylsphingosine or galactosylsphingosine.The formation of free sphingosine by enzymic splitting of glycosylsphingosine prior to the synthesis of cerebroside,was not required.

INTRODUCTION

Enzymic reactions for the N-acylation of sphingosine(1-3) and for the galactosylation and glucosylation of ceramide(4-8) have been established.These reactions are currently considered as parts of the pathways for the biosynthesis of cerebrosides and gangliosides.Enzymic reactions for the formation of galactosylsphingosine(9) and glucosylsphingosine(10,11) have also been described and consequently,these reactions offer the possibility of alternative pathways for those biosyntheses. In fact,the N-acylation of galactosylsphingosine,in an incubation system that contained rat brain microsomal preparation,was reported(12,13), but the reaction was nonenzymic(14).

In the present communication we report that when either glucosyl- or galactosylsphingosine was added to an incubation system which contained microsomal fraction from embryonic chicken brain and ^{14}C-stearoyl-CoA,the corresponding glycosylceramide was enzymically synthesized,and that the previous splitting of glycosylsphingosine to form free sphingosine was not required for the synthesis.

MATERIALS AND METHODS

Dowex 1-X8 (200-400 mesh) was purchased from Baker; 1-^{14}C-stearoyl-CoA was from New England Nuclear. DL-erythro-sphingosine was purchased from Miles. Glucosylsphingosine and Gaucher spleen glucosylceramide were prepared as previously described(10). Galactosylsphingosine and galactosylceramide (from bovine brain) were gifts from Dr. H.J. Maccioni and ceramide (stearoylsphingosine) was from Dr. F.A. Cumar. Galactosyl- and glucosylsphingosine were used as sulfate(15).

Enzyme preparation. Thirteen to fifteen-day-old embryonic chicken brains were homogenized with 4 volumes of 0.25 M sucrose containing 1.0 mM EDTA, pH 7.9. The suspension was centrifuged at 10,000 x g for 20 min and the precipitate homogenized and centrifuged again in the same conditions as before. The supernatant fractions were pooled and centrifuged at 12,000 x g for 30 min; the precipitate was discarded and the microsomal fraction was obtained from the supernatant by centrifugation at 100,000 x g for 60 min. The microsomal membranes from 5 g of brain tissue were suspended in 10 mM Tris-HCl buffer pH 7.55 to a final volume of 1.5 ml.

Incubation system and isolation of reaction products. Unless otherwise stated the incubation mixture contained, in a total volume of 60 µl, Tris-HCl buffer pH 7.55, 8 µmoles; 1-^{14}C-stearoyl-CoA, 1.9 nmoles (200,000 cpm); Tween 20, 60 µg; acceptor substrate, 0.1 µmole; and microsomal suspension, 20 µl. The incubation period was 20 min and the temperature 37°C. The reaction was stopped by the addition of 2.1 ml methanol and the mixture was heated at 55°C for 20 min and centrifuged. The extract was passed through a column containing 2 ml of packed Dowex 1 (Cl^- form) equilibrated with methanol-water 97:3 (v/v), and then eluted with the same solvent mixture. A total of 5 ml of effluent was collected and subjected to alkaline methanolysis(16). Glucosylceramide(0.12 µmole), ceramide(0.12 µmole), chloroform(9.5 ml), and water(2.5 ml) were added to the solution and the mixture was shaken. The aqueous phase

was separated and the lower phase was washed twice with theoretical upper phase(17) without salts.The washed lower phase was evaporated to dryness in vacuo and subjected to TLC on borate-containing silica gel-G with chloroform-methanol-water-ammonia 280:70:6:1 (v/v) (18) as solvent.Ceramide(Rf 0.75), glucosylceramide(Rf 0.56), and galactosylceramide(Rf 0.28) were run as standards.The plate was exposed to iodine vapor and the ceramide and glucosylceramide spots and the zone corresponding to galactosylceramide were scraped. The material obtained from each spot or zone was put in a separate column and eluted with 2 ml of chloroform-methanol-water 30:25:4 (v/v) followed by 2 ml of chloroform-methanol 2:1 (v/v).The effluent containing ceramide was collected in a vial,dried and counted. The effluents corresponding to glucosyl- and galactosylceramide were subjected to TLC on silica gel with chloroform-acetic acid-methanol 94:5:1 (v/v) as solvent; this system was used to eliminate some radioactive contaminants present in the cerebroside effluents.Glucosyl- and galactosylceramide were scraped from the plate,put in separate columns and eluted as before,and counted.

Characterization of the reaction products was as follows. For ceramide and glucosylceramide: the X-ray-film radioautogram obtained when the washed lower phase was run on borate plate (see above),showed radioactivity cochromatographing with the ceramide and glucosylceramide standards.For galactosylceramide and glucosylceramide: after isolation by TLC using the chloroform-acetic acid-methanol mixture as solvent,these compounds,mixed with the corresponding standard glycosylceramides,were subjected to TLC with chloroform-methanol-water 144:25:2.8 (v/v) as solvent; the radioautogram showed a radioactive spot in the zone of medium-chain NFA-cerebroside(5) of the respective standard stained with the anthrone reagent.

Radioactivity was measured in a Beckman LS-200 liquid scintillation spectrometer using a scintillator mixture containing 2 g PPO and 100 g naphthalene in 1000 ml dioxane.

Abbreviations: NFA, nonhydroxy fatty acid.

TABLE I

Conversion of 1-^{14}C-stearoyl-CoA to glucosylceramide, galactosylceramide, and ceramide.

Additions to the incubation mixture		^{14}C-stearoyl-CoA converted (cpm)					
		Glucosylcer.		Galactosylcer.		Ceramide	
Microsomal fraction	Acceptor	Exp.I	Exp.II	Exp.I	Exp.II	Exp.I	Exp.II
Active	Glucosylsphingosine	645	1320	85	210	6640	5237
None	Glucosylsphingosine	407				36	
Boiled	Glucosylsphingosine	10	20		15	35	20
Active plus boiled (*)	Glucosylsphingosine	305				3460	
Active	None	23		53		620	
Active	Galactosylsphingosine	115	160	3460	1950	5985	4490
None	Galactosylsphingosine		20		160		11
Boiled	Galactosylsphingosine		0		20		20
Active	Sphingosine(45 nmoles)		50		140		11748
Active	Sphingosine(5 nmoles)		100		120		10046
Active	Sphingosine(15 nmoles) plus glucose(15 nmoles)		80		150		15867
Boiled	Sphingosine(45 nmoles)		0		20		26

The incubation mixture contained in a total volume of 60 µl, 8 µmoles Tris-HCl buffer pH 7.55; 60 µg Tween 20; and 1.9 nmole 1-14C-stearoyl-CoA(200,000 cpm).Where indicated the amount of microsomal suspension and glycosylsphingosine(as sulfate) added was,respectively,20 µl and 0.1 µmole.Boiled microsomal fraction was obtained by heating the microsomal suspension in boiling water for 10 min.The incubation time was 20 min and the temperature 37°C.
(*) 20 µl of each microsomal suspension.

RESULTS AND DISCUSSION

Results in Table I show that glucosylceramide was formed during incubation of glucosylsphingosine with ^{14}C-stearoyl-CoA in the presence of microsomal fraction from embryonic chicken brain; however, since in the absence of the microsomal fraction a significant amount of glucosylceramide was also produced, it was of interest to ascertain whether or not the reaction was mostly enzymic. For this purpose we attempted the inhibition of the nonenzymic formation of glucosylceramide, based on the observation of Ullman and Radin(3) that mouse microsomal fraction inhibits the nonenzymic N-acylation of sphingosine by hydroxyacyl-CoA. When boiled instead of active microsomal fraction was used in our system, the amount of glucosylceramide formed decreased to 1-2% of that produced either in the presence or in the absence of active microsomal membranes. Incubation of glucosylsphingosine and ^{14}C-stearoyl-CoA with a 1:1 mixture of active and boiled microsomal fraction, lead to the production of 50% of the amount of glucosylceramide and ceramide formed when active microsomal fraction was added alone. This result indicates that in our system glucosylceramide was produced by enzymic synthesis, and suggests that the nonenzymic reaction was prevented by the binding of one or both of the substrates with the active as well as the boiled microsomal membranes, as was postulated by Ullman and Radin in their system.

The embryonic chicken brain microsomal fraction also catalyzed the formation of galactosylceramide when galactosylsphingosine was the acceptor added (Table I).

High amounts of ceramide were also produced in every case in which a glycosylceramide was enzymically formed (Table I). This suggested the possibility that the pathway to glycosylceramide was through ceramide rather than through glycosylsphingosine; ceramide could have been formed from free sphingosine added as an undetected contaminant of the glycosylsphingosine preparation, or released enzymically from glycosylsphingosine. The pathway through free

sphingosine was discarded, however, because addition of sphingosine or sphingosine plus glucose produced very high amount of ceramide but no glycosylceramide (Table I).

The overall conclusion of these results is that an enzymic reaction took place when microsomal fraction from embryonic chicken brain was incubated with stearoyl-CoA and either glucosyl- or galactosylsphingosine to produce a cerebroside; furthermore, since the glycosyl moiety of the cerebroside synthesized was in each case the same as that of the glycosylsphingosine added, it appears that the added glycosylsphingosine rather than any endogenous material was used for the synthesis. Nevertheless, crucial proof for this conclusion requires, besides a complete set of enzymic controls, that the synthesis be carried out with such labelled substrates as to allow the determination of isotopic distribution in the three moieties of the synthesized cerebrosides, as was suggested by Hammarström(13).

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