Occurrence of Glycosphingolipids in Chicken Egg Yolk

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Chicken egg yolk was found to contain a unique glycosphingolipid pattern not seen in other types of tissue or cell. These glycosphingolipids were isolated in pure form and their structures established by sequential enzymic hydrolysis and permethylation analysis. The major gangliosides in chicken egg yolk are *N*-acetylneuraminosylgalactosylceramide, *N*-acetylneuraminosyl-lactosylceramide and di-*N*-acetylneuraminosyl-lactosylceramide. The only neutral glycosphingolipid found in chicken egg yolk is galactosylceramide.

Glycosphingolipids occur in Nature mainly as constituents of biological membranes and other structural elements (Wiegandt, 1971). The specific patterns of glycosphingolipids have been shown to reflect tissue and species specificity (Hakomori, 1965; Wiegandt, 1971; Yamakawa, 1966). While studying the chemical composition of different components (shell and vitelline membranes, white and yolk) in chicken egg we found that only the yolk contains significant amounts of glycosphingolipids. Furthermore, these glycosphingolipids had a unique pattern not seen in any other type of tissue. The present paper describes the characterization of glycosphingolipids from the unfertilized chicken egg yolk.

Experimental

Materials

Neuraminidase (type VI) from *Clostridium perfringens* and DEAE-cellulose were purchased from Sigma (St. Louis, MO, U.S.A.). Silicic acid (Biosil A) was purchased from Bio-Rad (Richmond, CA, U.S.A.). We isolated β -galactosidase from jack bean (Li *et al.*, 1975), *N*-acetylneuraminosyl-lactosylceramide and glucosylceramide from human spleen (Svennerholm, 1963) and galactosylceramide from human brain (Radin, 1972). The following compounds were generously given: *N*-acetylneuraminosylgalactosylceramide from human brain (Ledeen *et al.*, 1973) was from Dr. R. K. Yu, Yale University, New Haven, CT, U.S.A., and di-*N*-acetylneuraminosyl-lactosylceramide from bovine retina (Holm *et al.*, 1972) was from Dr. M. Holm, University of Göteborg, Sweden.

Methods

Preparative or analytical t.l.c. of glycosphingolipids was performed as described by Svennerholm *et al.* (1973). Gangliosides and neutral glycosphingolipids were made visible by spraying the plates with resor-

cinol reagent (Svennerholm, 1957) and orcinol reagent (Svennerholm *et al.*, 1973). Sequential degradation of saccharide units in glycosphingolipids by exoglycosidases was carried out by the method of Li & Li (1976). Sphingosines were analysed after trimethylsilylation (Carter & Gaver, 1967), and fatty acids were separated on a DEGS-20 column as their methyl esters. Sialic acid species were determined by t.l.c. by the method of Granzer (1962).

Extraction of glycosphingolipids from chicken egg yolk

Twelve fresh unfertilized chicken eggs yolks were separated from the whites. The vitelline membranes were carefully removed by the procedure of Kido *et al.* (1975). The yolks were pooled, homogenized with 1 litre of acetone, and filtered through a Buchner funnel to obtain 90 g of acetone-dried powder. For extracting the glycosphingolipids, the acetone-dried powder was first homogenized with 360 ml of 0.01 M-KCl. To this homogenate we added 1440 ml of tetrahydrofuran and stirred the mixture at room temperature for at least 5 h. The residue after filtration on a Buchner funnel was re-extracted with 3 $\times$ 720 ml of tetrahydrofuran/water (8:1, v/v). All filtrates were combined and dried in a flash evaporator. To the dried residue, 180 ml of methanolic 0.6 M-NaOH was added and the mixture was incubated at 37°C for 6 h. After incubation, we added 90 ml of acetone and placed the mixture in a Revco deep-freezer (–60°C) overnight to precipitate glycosphingolipids. The precipitate obtained by filtration on a Buchner funnel was extracted successively with 180 ml each of chloroform/methanol (1:1, v/v) and chloroform/methanol (2:1, v/v). The extracts were combined and dialysed exhaustively against water, and dried in a flash evaporator to obtain the crude lipid extract.

DEAE-cellulose column chromatography

The crude lipid extract was dissolved in 40 ml of chloroform/methanol (4:1, v/v) and applied to a

column (30cm×2.9cm) of DEAE-cellulose (acetate form) that had been equilibrated with the starting solvent, chloroform/methanol/water (2:5:1, by vol.). After the column had been washed with 600 ml of the starting solvent, gangliosides were separated into two groups by eluting the column with the starting solvent containing 0.01 M- and 0.04 M-sodium acetate. Effluent from 0.01 M-sodium acetate (fraction I) contained monosialosylmonohexosylceramide and monosialosyldihexosylceramide, and disialosyldihexosylceramide was in the 0.04 M-sodium acetate eluate (fraction II). Further elution with solvent containing sodium acetate at a concentration higher than 0.06 M did not yield any higher gangliosides. Preparative t.l.c. was used to purify further the gangliosides in fractions I and II.

Silicic acid column chromatography

Silicic acid (Biosil A) was activated overnight at 110°C and packed into a column (23cm×1.9cm) with chloroform. The neutral glycosphingolipid fraction obtained from the DEAE-cellulose column was dissolved in 20 ml of chloroform and applied to this column. The column was first washed with 600 ml of chloroform to remove the neutral lipids, and subsequent elution with chloroform/methanol (19:1, v/v) yielded all the monohexosylceramide in the fraction. No higher neutral glycosphingolipid was detected when the column was further eluted with methanol. The monohexosylceramide was further purified by preparative t.l.c.

Results and Discussion

Gangliosides in chicken egg yolk

Of the various parts of the chicken egg, i.e. shell, shell membrane, vitelline membrane, egg white and egg yolk, only egg yolk contains a significant amount of glycosphingolipids. As Fig. 1 shows, the major gangliosides in chicken egg yolk are monosialosylmonohexosylceramide, monosialosyldihexosylceramide and disialosyldihexosylceramide. The chromatographic mobilities of these three gangliosides are identical with that of *N*-acetylneuraminosylgalactosylceramide isolated from human brain, *N*-acetylneuraminosyl-lactosylceramide isolated from bovine erythrocytes and di-*N*-acetylneuraminosyl-lactosylceramide isolated from bovine retina respectively. *N*-Acetylneuraminic acid was the only sialic acid found in these gangliosides. Monosialosylmonohexosylceramide and monosialosyldihexosylceramide were hydrolysed by neuraminidase to galactosylceramide and lactosylceramide respectively. The lactosylceramide derived from monosialosyldihexosylceramide was converted into glucosylceramide by jack-bean β -galactosidase. Disialosyldihex-

osylceramide was also converted into lactosylceramide by neuraminidase. During this enzymic hydrolysis *N*-acetylneuraminosyl-lactosylceramide was detected as an intermediate. The lactosylceramide derived from this ganglioside was in turn converted into glucosylceramide by jack-bean β -galactosidase. Permethylation (Yang & Hakomori, 1971) followed by g.l.c.-mass spectrometry of the partially methylated alditol acetates resulted in a 50-60% overall yield of 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetyl-galactitol from the monosialosylmonohexosylceramide and 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetyl-galactitol and 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetyl-glucitol from both monosialosyl- and disialosyl-dihexosylceramide. On the basis of the carbohydrate composition, sequential hydrolysis of saccharide units with exoglycosidases and the permethylation studies, the structures of these gangliosides were determined to be NeuAc α 2→3Gal→Cer, NeuAc α 2→3Gal β 1→4Glc→Cer and NeuAc2→?NeuAc α 2→3Gal β 1→4Glc→Cer. With the procedures described above we isolated 2.5 mg of *N*-acetylneuraminosylgalactosylceramide, 8.5 mg of *N*-acetylneuraminosyl-lactosylceramide and 1.5 mg of di-*N*-acetylneuraminosyl-lactosylceramide from twelve chicken eggs.

Neutral glycosphingolipids in chicken egg yolk

The only neutral glycosphingolipid found in chicken egg yolk is monohexosylceramide, which contains galactose as the sole neutral sugar. By using a borate-impregnated plate, the chromatographic

Fig. 1. T.l.c. of purified gangliosides isolated from chicken egg yolk

1, Egg yolk ganglioside mixture; 2 and 6, from top to bottom are: a, standard *N*-acetylneuraminosylgalactosylceramide; b, *N*-acetylneuraminosyl-lactosylceramide; and c, di-*N*-acetylneuraminosyl-lactosylceramide; 3, 4 and 5 correspond respectively to monosialosylmonohexosylceramide, monosialosyldihexosylceramide and disialosyldihexosylceramide isolated from chicken egg yolk. The plate was developed with chloroform/methanol/0.25% KCl (60:32:7, by vol.) and sprayed with resorcinol reagent.

Table 1. *Fatty acids and sphingosine composition of egg-yolk glycosphingolipids*

Abbreviations used: GM4, *N*-acetylneuraminosyl-galactosylceramide; GM3, *N*-acetylneuraminosyl-lactosylceramide; GD3, di-*N*-acetylneuraminosyl-lactosylceramide. d, Dihydroxy; t, trihydroxy.

	Content (%)			
	Gal-Cer	GM4	GM3	GD3
Fatty acid				
C _{16:0}	46	51	27	31
C _{16:1}	—	—	1	—
C _{18:0}	22	12	17	29
C _{18:1}	28	30	47	30
C _{18:2}	4	1	1	—
C _{20:0}	—	—	1	—
C _{21:0}	—	5	1	2
C _{22:0}	—	—	3	2
C _{22:1}	—	—	—	3
C _{23:0}	—	—	—	2
C _{24:0}	—	—	—	2
Sphingosine				
dC _{18:1}	93	85	58	73
dC _{18:0}	3	10	15	16
tC _{18:0}	4	5	29	11

mobility of this glycolipid is identical with that of galactosylceramide isolated from human brain. Permethylation analysis of this glycolipid revealed a single sugar peak, corresponding to 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl-galactitol. Twelve chicken eggs yield about 7.5 mg of this glycolipid.

Sphingosine and fatty acid compositions

As Table 1 shows, the major fatty acids of egg glycosphingolipids are palmitic acid, stearic acid and oleic acid, and C₁₈ sphingosine is the predominant long-chain base in these glycolipids. It is noteworthy that *N*-acetylneuraminosyl-lactosylceramide and di-*N*-acetylneuraminosyl-lactosylceramide contain appreciable amounts of sphinganine and phytosphingosine. In contrast with the galactosylceramide from human brain, no α -hydroxy fatty acid was detected in the galactosylceramide isolated from chicken egg yolk.

Since chicken egg yolk is very rich in cholesterol and cholesteryl esters, as well as other lipids, we used cold acetone to precipitate the glycosphingolipids after saponification of the total lipid extract. We found that this step greatly facilitates the subsequent purification of gangliosides and neutral glycosphingolipids by DEAE-cellulose chromatography.

Avian eggs are specialized cells that differ considerably from other types of cell. *N*-Acetylneuraminosylgalactosylceramide has been considered to be a myelin-specific ganglioside (Ledeen *et al.*, 1973). Di-*N*-acetylneuraminosyl-lactosylceramide, on the

other hand, has been found to be the major ganglioside in retina (Holm *et al.*, 1972), and *N*-acetylneuraminosyl-lactosylceramide the predominant ganglioside in visceral organs. It is intriguing to find these gangliosides in chicken egg yolk. The major neutral glycosphingolipid in egg yolk is galactosylceramide, which is the major glycosphingolipid of normal adult brain. The presence of galactocerebroside in egg yolk was reported by Levene & West (1917). Since the egg yolk possesses all the potentialities for embryonic development and differentiation, the unique glycosphingolipid pattern in egg yolk may have special and as yet unknown physiological function. It should be pointed out that the availability of chicken eggs make them a convenient source for the above-mentioned glycosphingolipids.

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