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Isolation of Bovine Kidney Gangliosides

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A controlled procedure for quantitative extraction and isolation of five pure gangliosides (I—V) of bovine kidney is described. The carbohydrate determinations and thin layer chromatographic behaviour of the isolated gangliosides suggest that gangliosides I and II (together 72 % of total) are hematosides, III (22 %) is a ceramide-dineuraminyl-dihexoside and IV and V (together 6 %) are more complex polysialogangliosides.

The cerebral gangliosides have been extensively studied.[1] The amount of neuraminic acid containing glycolipids in other tissues is relatively low,[2] and only few investigators have paid attention to extraneural gangliosides. However, during the last few years the findings that gangliosides are haptens,[3] and the hypothesis about their role in ion transport,[4] and as serotonin receptor [5] have aroused interest in these complex glycolipids also outside central nervous system.

This paper describes the isolation of five gangliosides (I—V) of bovine kidney. The dominating role of non-polar gangliosides in the complex ganglioside pattern of kidney was demonstrated in our previous communication.[2] The available methods [6–9] suitable for the study the more polar cerebral gangliosides therefore required considerable modifications to give reliable results in the extraction and purification of kidney gangliosides. To prevent selective losses of different gangliosides, the yield of lipid bound neuraminic acid, and the behaviour of individual gangliosides were analyzed for every step of the isolation procedure. The thin layer chromatographic properties, quantitative distribution and some structural characteristics of the isolated fractions are presented.

The findings are related to the existing information about the kidney gangliosides arising from the studies concerning mainly non-gangliosidic renal glycolipids.[10–13]

MATERIALS AND METHODS

Materials

The bovine kidneys were obtained from healthy animals and used immediately. The brain gangliosides used as reference in thin layer chromatography were made available by Professor E. Klenk (Physiologisch-Chemisches Institut der Universität Köln, Cologne, Germany).

Adsorbents for chromatography were silicic acid (Anasil-S, Analytical Engineering Laboratories, Inc., Hamden, Conn.), cellulose (Whatman powder, standard grade, W & P, Balston, Ltd.) and silica gel G (E. Merck, AG., Darmstadt). Ion exchange resins used were Chelex-100, 100–200 mesh, Bio Rad Laboratories (Richmond, Calif.) and Dowex 50×8 (200–400 mesh) Fluka AG. (Buchs, S.G., Switzerland). Organic solvents were of analytical reagent quality and were distilled before use.

Analytical methods

Quantitative analytical methods. Neuraminic acid from lipid extracts was determined according to Svennerholm,[14,15] for crude lipid extracts ion exchange purification was used.[16] Neuraminic acid from thin layer plates was analysed directly as described by Suzuki.[17] Free neuraminic acid was determined by the method of Warren.[18] *N*-Acetylneuraminic acid (Sigma Chemical Company, St. Louis, Missouri) was employed as standard in all determinations. The hexoses were measured by a modified anthrone reaction [19] and the hexosamines by a modified Elson-Morgan reaction.[20] Sphingosines were determined after hydrolysis (2 M HCl, 2 h, 100°C) according to Lauter [21] using sphinganine as standard. Phosphorus was analysed according to Itay and Ui.[22] To obtain dry weight values, tissue samples were dried to constant weight at 100°C.

Thin layer chromatography (TLC). Silica gel was used as adsorbent. The TLC plates were prewashed in methanol/ether mixture (4:1) * and activated for 1 h at 110° before use. Either resorcinol [14] or sulphuric acid spray were used for the detection. The solvents were: A) Chloroform/methanol/water (60/35/8) B) Chloroform/methanol/2.5 M NH_4OH (60/35/8) C) propanol/water (7/3) D) propanol/water/concentrated ammonia (6/2/1).[23]

Preparative TLC was carried out with 1 mm plates. The ganglioside fractions were detected both under ultraviolet light and by resorcinol spray of the silica gel that had been attached to a strip of adhesive tape placed in the middle of the plates.[24] The gangliosides were eluted from silica gel by shaking the powder twice with a methanol/chloroform/pyridine/water (4/2/1/1) mixture.

Determination of proportions of different gangliosides in lipid extracts. A band of lipid extract containing a known amount of neuraminic acid was pipetted on a precoated thin layer plate (DC-Fertigplatten F_{254}, E. Merck, Darmstadt, Germany) with reference spots of the same extracts on both ends of this band. The plate was run in solvent C and the narrow strips containing the reference spots were cut off and sprayed with resorcinol. Three fractions were separated reliably. On the basis of reference strips, the fractions were scraped off from the unsprayed plate and the neuraminic acid content was determined.[17] The fast moving fraction contained the gangliosides I and II, the intermediate fraction ganglioside III and the slow moving fraction gangliosides IV and V.

EXPERIMENTAL AND RESULTS

A schematic presentation of the preparation procedure is given in Fig. 1. To prevent auto-oxidation, BHT (di-*tert*-butyl-*p*-cresol) was added to organic solvents.[25] All column chromatographic runs were performed at +4°C. The individual gangliosides were designated I, II, III, IV, and V in order of decreasing TLC mobility.

* All solvent ratios are expressed as volume units.

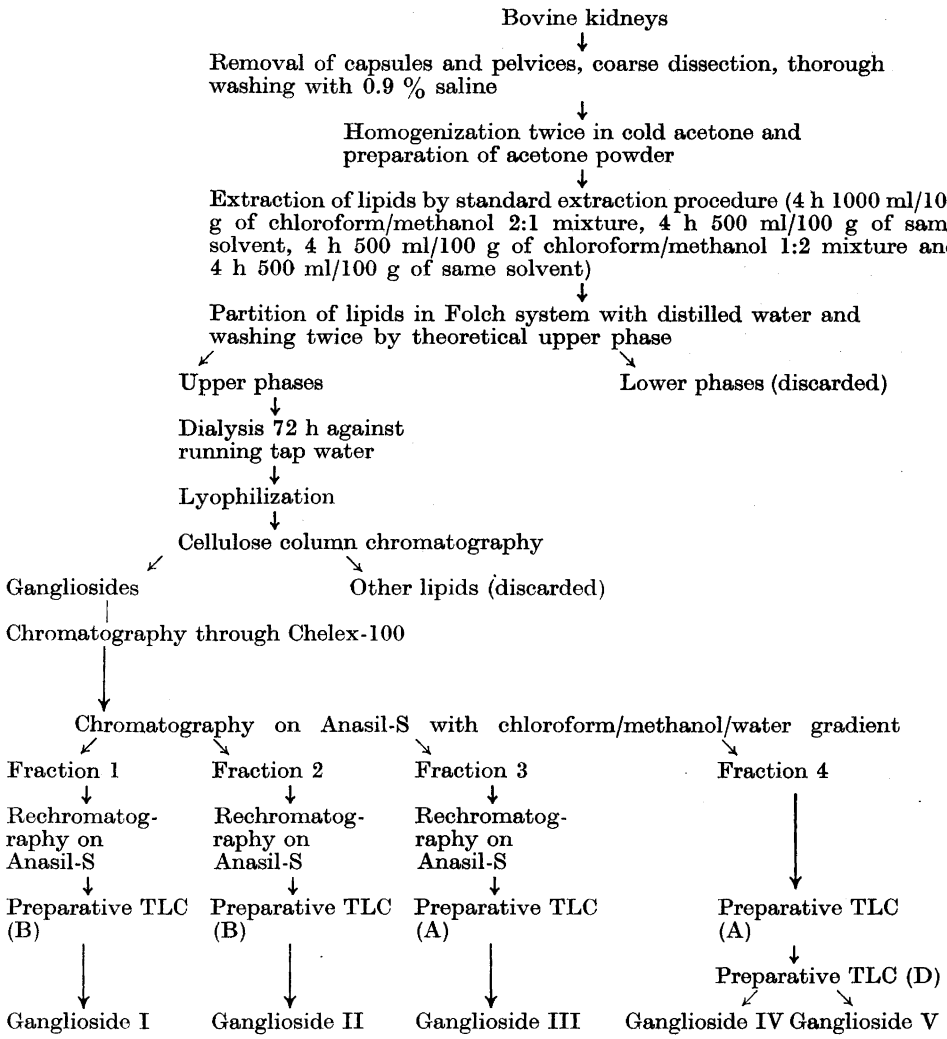

Fig. 1. Diagramatic representation of the isolation procedure. Letters in parentheses denote the solvent systems used in the preparative thin layer chromatography.

Preparation of acetone powder

After rough cleansing the kidneys were made bloodless by arterial perfusion and by thorough rinsing of minced tissue with 0.9 % NaCl. The analysis of rinsing solution for lipid bound neuraminic-acid, revealed that the extensive washing did not extract the water soluble gangliosides. The rinsed tissue was homogenized twice in cold acetone and dried to acetone powder. The acetone

treatment extracted a small amount of lipid bound neuraminic acid (about 0.01 mg/100 g dry weight) mainly gangliosides I and II. This is less than 0.1 % of the total lipid bound neuraminic acid.

Extraction of lipids

The extraction of the acetone powder by the chloroform/methanol 2:1 mixtures (twice for 4 h, 1000 ml and 500 ml/100 g acetone powder) removed only 80 % of total extractable lipid bound neuraminic acid. These extracts contain mainly the nonpolar gangliosides I and II (Table 1). Further extraction of the residue with chloroform/methanol 1:2 mixture (twice for 4 h, 500 ml/100 g acetone powder) was essential for maximal yield of the polysialogangliosides (gangliosides III, IV, and V) (Table 1). Neither the prolongation

Table 1. Analysis of gangliosides extracted by different solvents.

Extraction procedure	Total lipid bound neuraminic acid extracted	Percent distribution of lipid bound neuraminic acid		
		I, II	III	IV, V
First chloroform/methanol 2/1	9.48	75	21	3
Second chloroform/methanol 2/1	2.21	71	26	1
Third chloroform/methanol 2/1	0.42	—	—	—
First chloroform/methanol 1/2	2.97	24	54	21
Second chloroform/methanol 1/2	0.92	19	57	22
Third chloroform/methanol 1/2	0.01	—	—	—

Lipids were extracted from the same sample of acetone powder with successive treatment with chloroform/methanol mixtures. Total lipid-bound neuraminic acid and percentual distribution were determined by methods described in the text.

nor the further repetition of the extraction with this mixture increased the yield of lipid bound neuraminic acid. The use of more polar solvent mixtures resulted in an appreciable extraction of non-lipid material and did not improve the yield of gangliosides. No further lipid bound neuraminic acid could be extracted from acetone powder with hot solvents in the Soxhlet apparatus. This treatment resulted in a degradation of the polysialogangliosides.

Thus the standard extraction procedure was established as two extractions at room temperature with chloroform/methanol 2:1 followed by two more with chloroform/methanol 1:2 (Fig. 1).

Chloroform was added to the combined extracts to give the proper ratios of the solvents (2:1) for the Folch partition. Thereafter the final volume of the crude lipid extract was 3500 ml/100 g acetone powder.

Purification of gangliosides from other lipids

The separation of gangliosides from the crude extract did not succeed by the classical Folch partition using salt solution.[25] The presence of salt in the upper phase during the Folch partition resulted in a considerable loss of the nonpolar gangliosides I and II to the lower phase (Table 2).

Table 2. Analysis of gangliosides obtained by different partition procedures.

		Total lipid bound neuraminic acid	Percentual distribution of lipid bound neuraminic acid		
			I, II	III	IV, V
Partition with deionized water	Upper phases	14.22	63	29	8
	Lower phases	1.88	61	31	9
Partition with 0.1 % KCl	Upper phases	8.92	43	45	12
	Lower phases	7.28	83	13	14

Two lipid extracts (3 600 ml) obtained by standard extraction procedure (16.20 mg lipid-bound neuraminic acid in each) were partitioned with deionized water and 0.1 % KCl (600 ml). Both upper and lower phases were analyzed for lipid-bound neuraminic acid and the percentual distribution of gangliosides was determined as described in the text.

The use of deionized water in the Folch partition produced the maximal partition of the kidney gangliosides into the upper phase (Table 2). All lipid bound neuraminic acid was partitioned to the aqueous phases after two washings with the theoretical upper phase. Separate experiments showed that significant reduction of the volume of the crude lipid extract before the partition leads to considerable loss of gangliosides into the chloroform phase.

After the Folch partition the combined upper phases were dialyzed for 72 h against running tap water. The amount of lipid bound neuraminic acid and the TLC patterns of gangliosides did not change during the dialysis. The dialyzed upper phases were lyophilized and the resulting powder was dissolved in a small amount of chloroform/methanol 2:1.

Unfortunately, the use of deionized water in the Folch procedure results in the partition of appreciable amounts of other lipids into the upper phases.[26] The content of lipid bound neuraminic acid was 2 % of the total solids in the dialyzed upper phases, corresponding approximately to 8 % of gangliosides. Hence, the further purification of gangliosides from these other lipids was necessary before the fractionation of the ganglioside mixture into the individual components. Cellulose column chromatography was used for the separation of gangliosides from the other lipids in the dialyzed upper phases of modified Folch partition. The cellulose column chromatography was carried essentially according to Rouser *et al.*[27] Usually about 40 mg gangliosides was transferred on the cellulose column (2.5 cm × 30 cm, 20 g cellulose powder). The other lipids were eluted with chloroform/methanol 9/1 saturated with water (1400 ml) and thereafter the total bulk of gangliosides with methanol/water 9:1

(800 ml). The recovery of the gangliosides was 98 %. The content of lipid bound neuraminic acid was 22 % of the total solids in the methanol/water eluate, corresponding approximately to 88 % of gangliosides. The neuraminic acid determinations and TLC revealed that about 1 % of gangliosides I and II were eluted with the other lipids. The mixed ganglioside fractions still contained about 0.2 % phosphorus, and TLC revealed the presence of unidentified neuraminic acid free lipids in this fraction.

Separation of the individual gangliosides

Because of the marked differences in the solubility of various salts of acidic lipids [28,29] the mixed gangliosides obtained from the cellulose column were converted to sodium salts by Chelex-100 ion exchange chromatography according to Carter *et al.*[28]

The sodium salts of gangliosides (about 80 mg) were transferred on to the Anasil-S column (60 g, diameter to length ratio 1 to 20) [23] and the elution of individual gangliosides was carried out by chloroform/methanol/water gradient with steadily increasing polarity. The solvent gradient was obtained using a two-stage closed-vessel (1000 ml) apparatus, with constant stirring of the lower reservoir. At the beginning of the run the solvent mixtures in the lower and upper reservoirs were chloroform/methanol/water 70/34/5 and 60/30/5, respectively. After the upper reservoir had emptied 1000 ml of chloroform/methanol/water 55/35/8 mixture was added. Fractions of 10 ml were collected and 0.1—0.5 ml from each fraction were taken for neuraminic acid determinations and TLC analysis.

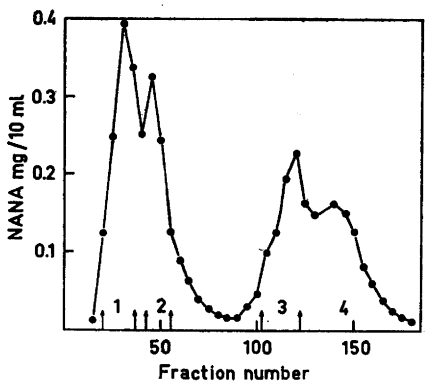

Fig. 2. Chromatography of mixed ganglioside (80 mg) on Anasil-S column. The elution was carried out with a continuous gradient from 70/34/5 to 55/35/8 chloroform/methanol/water (v/v/v). Peaks 1—4 were pooled on the basis of neuraminic acid determinations and thin layer chromatographic analysis in four solvent systems.

Four fractions (fraction 1, 2, 3, 4) were consistently separated by silicic acid chromatography (Fig. 2). These crude fractions were further purified by rechromatography and preparative TLC.

Fraction 1. This fraction contained chiefly the most nonpolar ganglioside I. The material obtained by several silicic acid chromatographies was pooled and further separated from the contaminating fraction 2 by rechromatography on silicic acid. The final purification of ganglioside I was accomplished by

preparative TLC in solvent B. The ganglioside I, already homogeneous in TLC, was deionized by Dowex-50 (H^+) and lyophilized. The white powder thus obtained was dissolved in methanol and precipitated by adding ether. The precipitate was dissolved in a small quantity of methanol, and the gangliosides were again precipitated at +4°. The homogeneity of ganglioside I was confirmed by TLC on four solvent systems (Figs. 3—6). This ganglioside moved faster than the brain monosialoganglioside (GM_1) * in all solvent systems, and its relative mobility was identical with that of neuraminyllactosylceramide as reported by Penick *et al.*[23]

The photometric analysis revealed that the ratio of hexose:neuraminic acid:sphingosine in ganglioside I was 2.12:0.94:1.00.

Fraction 2. This fraction consisted mainly of ganglioside II but also contained considerable amounts of ganglioside I. The material obtained from

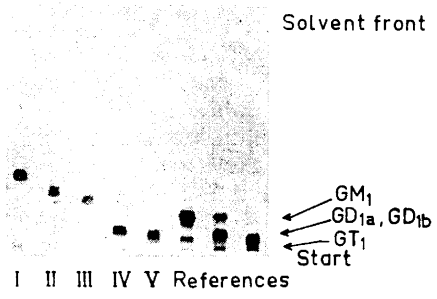

Fig. 3. Thin layer chromatogram of bovine kidney gangliosides I—V. Solvent: chloroform/methanol/water 60:35:8. Spray: resorcinol. Brain gangliosides are run as references. The nomenclature of Svennerholm [30] is used.

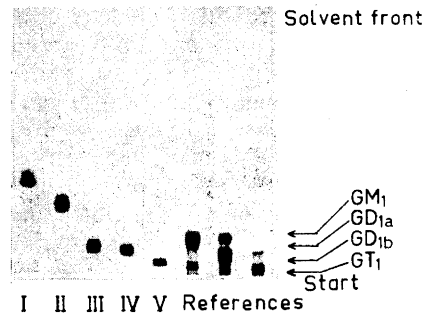

Fig. 4. Thin layer chromatogram of bovine kidney gangliosides I—V. Solvent: chloroform/methanol/2.5 M NH_4OH 60:35:8. Other conditions as for Fig. 3.

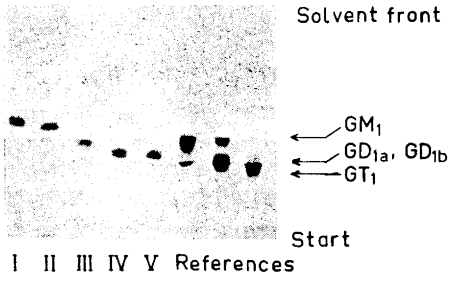

Fig. 5. Thin layer chromatogram of bovine kidney gangliosides I—V. Solvent: propanol/water 7:3. Other conditions as for Fig. 3.

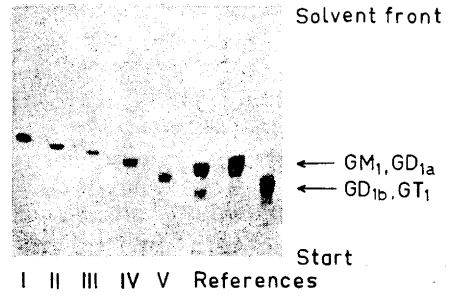

Fig. 6. Thin layer chromatogram of bovine kidney gangliosides I—V. Solvent: propanol-water-conc. NH_4OH 7:2:1. Other conditions as for Fig. 3.

* The nomenclature of Svennerholm [30] is used in the text.

several silicic acid chromatographies was pooled and rechromatographed on silicic acid. The ganglioside II was finally purified by preparative TLC in solvent B, deionized and precipitated as described before.

Ganglioside II moved faster than GM_1 but slower than ganglioside I in all four solvent systems. The best separation of the two nonpolar gangliosides (I and II) was achieved in chloroform/methanol/water solvents (A and B), (Figs. 3—6).

The ratio of hexose:neuraminic acid:sphingosine in ganglioside II was 1.87:2.06:1.00.

Fraction 3. This fraction was mainly composed of ganglioside III, with small amounts of gangliosides IV and V. Pooled material of several silicic acid chromatographies was rechromatographed on silicic acid, and the ganglioside III was further purified by preparative TLC in solvent A. Finally it was deionized and precipitated as described above.

The TLC properties of this ganglioside were unusual. In solvents B and C it moved slower, but in A and D faster than GM_1. In all these solvents ganglioside III moved slower than gangliosides I and II (Figs. 3—6).

The ratio hexose:neuraminic acid:sphingosine in the ganglioside III was found to be 2.04:1.88:1.00.

Fraction 4. This was heterogeneous and contained equal amounts of gangliosides III and IV, and a smaller amount of ganglioside V. Ganglioside III was removed from gangliosides IV and V by preparative TLC in solvent B. Gangliosides IV and V were further separated from each other by preparative TLC in solvent D.

The TLC properties of gangliosides IV and V resembled in those of GD_{1a} and GD_{1b} of brain, respectively, in all the solvent systems (Figs. 3—6).

The ratios of hexose:hexosamine:neuraminic acid:sphingosine is 3.12:1.04:1.87:1.00 in ganglioside IV and 2.87:0.89:3.14:1.00 in ganglioside V.

None of the purified gangliosides contained measurable amounts of phosphorus.

Quantitative distribution of gangliosides in the kidney

The total amount of lipid bound neuraminic acid in the kidney was 16 mg/100 g dry weight. The percentual distribution of the five kidney gangliosides was estimated after cellulose column chromatography. The mixed gangliosides were separated by TLC, and the neuraminic acid content of each fraction determined. On the basis of the carbohydrate analysis, and assuming that each ganglioside contains one molecule of sphingosine (C-18) and one molecule of stearic acid, the percentual distribution of different gangliosides in bovine kidney is: I 47 %, II 25 %, III 22 %, IV 4 % and V 2 %.

DISCUSSION

The use of chloroform/methanol 1:2 mixture in addition to the conventional, less polar chloroform/methanol 2:1 mixture proved necessary for quantitative extraction of neuraminic acid containing glycolipids from bovine kidney

acetone powder. Although determination of the percentual distribution of individual gangliosides is disturbed by the impurities in crude lipid extracts, it was clearly shown that the use of chloroform/methanol 1:2 is necessary for reliable extraction of polar polysialogangliosides. This fact has been previously observed in brain tissue.[6]

The failure of the classical Folch partition using salt solutions to separate the gangliosides from other lipids is probably best explained by the high content of the less polar gangliosides in kidney. This assumption is supported by the fact that especially gangliosides I and II were not extracted by salt solutions. The poor yield of nonpolar gangliosides with salt solution has been demonstrated previously.[29,31,32] The use of salt solution was primarily introduced by Folch *et al.* to minimize the loss of other lipids into the upper phase.[26] Thus, it is only natural that the use of deionized water resulted in the partition of considerable amounts of impurities into the upper phase. Further purification of the ganglioside mixture by cellulose column before the silicic acid chromatography was therefore essential.

There are some previous reports about the presence of neuraminic acid containing glycolipids in kidney.[10–13] However, thus far, only one ganglioside has been purified from this tissue and this ganglioside was found to contain glucose:galactose:neuraminic acid in a ratio 1:1:1.[13] On the basis of TLC Mårtensson *et al.*[11] proposed that one of the human kidney gangliosides is a hematoside.

The composition of carbohydrates and TLC properties of gangliosides I and II of present study are in accordance with the structure of hematoside, isolated from several other tissues.[32–40] The presence of a component identical to ganglioside III in regards to the molar ratios of monosaccharides has been demonstrated in human brain.[41] The ratios of carbohydrates in the gangliosides IV and V are in accordance with the analytical results of the di- and trisialogangliosides isolated from brain.[8]

Apart from the central nervous system, information on the quantitative distribution of gangliosides is available only from adrenal medulla,[32] where hematosides form 92 % of their total amount. According to the present study the percentage of hematosides in kidney is relatively high, 72 %, although appreciably lower than in adrenal medulla.

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