Rabbit muscle gangliosides

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Abstract Four ganglioside fractions were isolated from rabbit muscle: one hematoside and three hexosamine-containing species. They were analyzed for hexoses, hexosamine, sialic acid, fatty acids, and long-chain base content. The molar ratios of sphingosine–hexose–hexosamine–sialic acid were: for hematoside, 1:2:0:1; for the disialogangliosides, 1:3:1:2; and for trisialoganglioside, 1:3:1:3.

The carbohydrates were studied by thin-layer and paper chromatography. The hexoses were glucose and galactose; the hexosamine was N-acetylgalactosamine and the sialic acid was N-acetylneuraminic acid.

Fatty acids and long-chain bases were analyzed by gas-liquid chromatography. The fatty acid composition was similar in all of the four gangliosides. The most abundant fatty acids were 16:0 and 18:0, but significant amounts of 16:1, 18:1, 20:0, and 22:0 were also found. Hydroxy fatty acids were not detected. In all of the muscle gangliosides the main long-chain bases were C_{18}-sphingenine and C_{20}-sphingenine. In hematoside there were also measurable amounts of C_{18}-sphinganine and C_{20}-sphinganine, whereas in the major gangliosides only traces of C_{18}-sphinganine were detected.

Supplementary key words neutral hexoses · N-acetylgalactosamine · N-acetylneuraminic acid · fatty acids · long-chain bases

THE ISOLATION of gangliosides from muscle of man, rat, and rabbit was first reported by Lassaga, Albarracin, and Caputto (1). More recently, the presence of gangliosides in several extraneural tissues of rat, rabbit, and pig was described (2), and Max, Nelson, and Brady (3) reported that in denervated skeletal muscles the hematosides increase in relation to the control level. The amount of gangliosides found in muscles, which is in most cases about 0.05 μmole of NANA/g of fresh tissue (1), is low in relation to that found in brain gray matter or peripheral nervous tissue; the concentration of gangliosides in these tissues ranges from 0.8 to 1.5 mg/g of fresh tissue. The pattern of relative concentrations of different gangliosides found in muscle (1) is similar to that recently found in bovine adrenal medulla (4) and in bovine kidney (5). In all of these extraneural tissues, the hematoside MG-1 type of ganglioside prevails quantitatively over the more complex gangliosides, whose concentrations are about 10 to 20-fold lower than that of the hematoside.

Probably due to the small amounts available and the difficulties inherent in the purification of these compounds which are in very low concentration, practically nothing is currently known about the complex gangliosides from muscle and other tissues. This report deals with the composition and some of the properties of the complex gangliosides isolated from muscle.

MATERIALS AND METHODS

Pure grade solvents from Atanor Co. (Argentina) were used; for chromatographic separations these solvents were purified by distillation. Florisil was from the Floridin Co., Tallahassee, Fla. Silica gel G, D-glucose, and D-galactose were from E. Merck, Darmstadt, Germany. N-Acetylneuraminic acid, N-glycolylneuraminic acid, D-glucosamine, D-galactosamine, and glucose oxidase were from Sigma Chemical, St. Louis, Mo. Neuraminidase type IV (from *Clostridium perfringens*) was purchased from Worthington Biochemical, Freehold, N.J. Tetrahydrofuran and fatty acids were from Eastman Organic Chemicals, Rochester, N.Y. Sphingosine and dihydrosphingosine were from Miles Laboratories, Elkhart, Ind.

Abbreviations: NANA, N-acetylneuraminic acid; NGNA, N-glycolylneuraminic acid; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; LCB, long-chain bases. Symbols used for muscle gangliosides were based on the mobility on TLC: hematoside, MG-1; disialogangliosides, MG-2 and MG-3; trisialoganglioside, MG-4. For brain gangliosides the symbols proposed by Svennerholm (6) were adopted: monosialoganglioside, G_{M1}; disialoganglioside, G_{D1a}; disialoganglioside, G_{D1b}; trisialoganglioside, G_{T1}.

Analytical methods

Sphingosine was determined by the method of Lauter and Trams (7), using either dihydrosphingosine or sphingosine as standard. Total hexosamines were determined according to Svennerholm (8), and gangliosides were quantified by their total sialic acid with the orcinol reagent by the method of Böhm, Dauber, and Baumeister (9). When lesser amounts of material were available, the sialic acid was determined after hydrolysis in 0.1 N H_2SO_4 at 80°C for 2 hr by the method of Aminoff (10); in these cases a factor of 1.3 was applied to compensate for the fraction destroyed during hydrolysis plus the nonhydrolyzed sialic acid (11). The glycolyl groups were determined by the method of Klenk and Uhlenbruck (12). Acyl groups were measured by the hydroxamic acid method of Hestrin (13) and total hexoses with anthrone by the method of Trevelyan and Harrison (14) or by copper reduction according to Nelson (15). Glucose was quantified enzymatically by using glucose oxidase (16). Fatty acid determinations were carried out by GLC, using an F & M model 400 instrument equipped with a flame ionization detector. Glass U columns, 1.80 m × 3 mm I.D., were packed with 10% EGSS-X on 80–100 mesh, acid washed, dimethyl-dichlorosilane-treated Chromosorb W (Applied Science Laboratories, State College, Pa). The column was operated at 180°C with nitrogen as carrier gas. For hydroxy fatty acids a column of 3% SE-30 (Applied Science Laboratories) operated at 225°C was used. Areas were determined by multiplication of peak height by width at half height. Identification of the different fatty acids was done by comparison with samples of commercial origin (Eastman).

Extraction of gangliosides

Hind leg and back muscles (100 g) from an adult rabbit were used. In preliminary assays, various methods for extraction of gangliosides were compared: Klenk (17), Sweeley and Walker (18), Folch, Lees, and Sloane Stanley (19), and Trams and Lauter (20). The recovery of total gangliosides was estimated by the total NANA content. Using tetrahydrofuran and phosphate buffer (20), almost twice as much NANA was extracted as when the method of Folch et al. (19) was used (extraction with a mixture of chloroform–methanol 2:1 followed by partition with 0.2 vol of 0.88% KCl). When the extraction with chloroform–methanol was followed by partition with 0.2 vol of distilled water, the yields of both procedures (19, 20) were approximately the same. The other methods assayed yielded less gangliosides than either of the two previously mentioned.

Separation of individual gangliosides

The tetrahydrofuran extract was filtered through paper, and the filtrate was partitioned with ethyl ether (tetrahydrofuran–ether 15:4.5). After 4–5 hr, the lower (aqueous) phase was separated from the upper phase; the upper phase was washed twice and the lower phases were pooled and evaporated to approximately 50 ml. This concentrated solution was dialyzed against running tap water for 18 hr, and then against distilled water for 12 hr. The solution inside the dialysis tubing was evaporated to dryness and the dried material was dissolved in chloroform–methanol–water 10:10:3. The solution was analyzed for NANA. Determination with the orcinol reagent (9) showed that 1.5 mg of gangliosidic NANA was obtained from 100 g of muscle. The mixture of gangliosides was passed through a column of Florisil according to the method of Kishimoto and Radin (21) as modified by Rouser (22). The fraction eluted with chloroform–methanol 2:1 contained most of the MG-1, whereas the one eluted with methanol contained the remaining gangliosides. These two fractions were run on preparative TLC plates using chloroform–methanol–10% ammonia 60:35:8 as solvent. The plates were sprayed with water and the lipid spots were marked; in addition, a band of approximately 1.5 cm was cut from one of the edges, sprayed with resorcinol reagent, and heated for identification of sialic acid. The zones containing lipids and sialic acid were scraped from the plates and eluted with chloroform–methanol–water 10:10:3 (4), and the lipids were chromatographed again on TLC. To verify the purity of a spot, two bands of approximately 1 cm each were sprayed, one with 50% sulfuric acid and the other with resorcinol reagent (23). A ganglioside was considered pure when a single spot for each spray in the same position in both bands was obtained. The solvent systems used were chloroform–methanol–10% ammonia 60:35:8 (24) and n-propanol–water 7:3 (25).

Separation of ganglioside components

For fatty acid determinations, samples of the material eluted from each spot and containing 0.2 µmole of NANA were suspended in 3 N HCl in anhydrous methanol and methanolyzed in sealed tubes in a boiling water bath for 8 hr. Hydrolysis for sphingosine determination was carried out in hydrochloric acid–water–methanol 8.6:9.4:82 for 18 hr at 70°C (26), and hydrolysis for carbohydrate determinations was carried out in 3 N HCl for 8 hr. The hydrolyzed material was evaporated under vacuum, dissolved in 1 ml of distilled water, and partitioned with 1 ml of chloroform. The upper water phase was passed through a Dowex 50 (H$^+$) column to separate hexoses from hexosamines; hexosamines were eluted from the column with 2 N HCl. The lower, chloroform phase, which contained sphingosine and fatty acid methyl esters, was evaporated to

dryness and the fatty acid methyl esters were separated from sphingosine by dissolving them in cold hexane. Before analysis, the hexosamines eluted from Dowex 50 (H$^+$) were acetylated according to Carlson, Swanson, and Roseman (27) and passed again through Dowex 50 (H$^+$); N-acetyl hexosamines were subjected to TLC in borate-treated silica gel G, using n-propanol–water 7:3 as solvent, according to Gal (28). To locate the sugars, the plates were stained with ammonium bisulfate according to Ziminski and Borowski (29).

RESULTS

The chromatographic pattern

Fig. 1 shows the chromatographic pattern of the mixture of gangliosides of rabbit muscle run on TLC with solvent chloroform–methanol–10% ammonia 60: 35:8 compared with the pattern of the gangliosides from rabbit brain. It is noticeable that in muscle the major ganglioside is MG-1, followed by MG-3, MG-2, and MG-4, whereas in brain the main ganglioside is G_{D1a}, followed by G_{M1}, G_{D1b}, and G_{T1}.

Compositions of the different gangliosides

Hexoses. The determination of hexoses consistently produced higher values than expected for the gangliosides tentatively identified as MG-3 and MG-4. The experimental ratios of hexose to sphingosine were, respectively, 3.26 and 3.27; despite repeated purifications we could not eliminate the contaminant that apparently enhanced the carbohydrate reaction (Table 1).
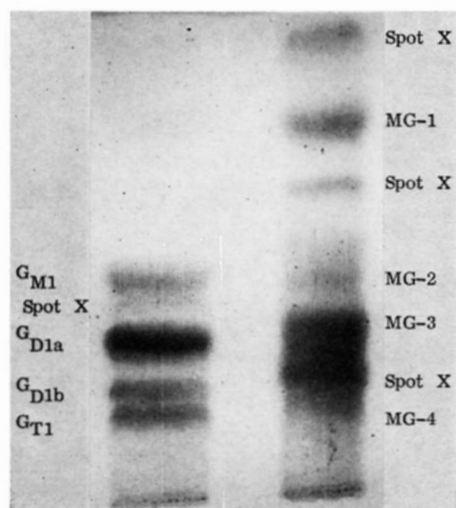

FIG. 1.   Thin-layer chromatograms of crude brain (*left*) and muscle (*right*) rabbit gangliosides. Solvent, CHCl$_3$–CH$_3$OH–10% NH$_4$OH 60:35:8; spray, resorcinol–HCl (23). Spots X were brown in color and did not belong to any gangliosides. No attempt was made to eliminate the brown spots because procedures to purify gangliosides as a total altered the relative amounts of them with a relative decrease of the hematoside.

TABLE 1.   Molar ratios of constituents of rabbit muscle gangliosides

Ganglio-sides	Sphin-gosine[a]	Total Hexoses[b]	Galactose/ Glucose[c]	Hexosamine[d]	NANA[e]
MG-1	1	2.14	0.97		1.07
MG-2	1	3.04	2	0.92	2.17
MG-3	1	3.26	2.25	0.93	2.02
MG-4	1	3.27	2.22	0.92	2.99

Results are expressed as molar ratios to sphingosine.
[a] Sphingosine was determined with methyl orange (7).
[b] Total hexoses were determined by the anthrone method (14).
[c] Glucose and galactose values were obtained by difference between the total reducing power and that remaining after treatment with glucose oxidase (16).
[d] Hexosamines were determined by the method of Svennerholm (8).
[e] NANA was determined by the orcinol method (9).

The values of hexoses and sphingosine found for MG-1 were in good agreement with a 2:1 ratio. Glucose and galactose in MG-1 were determined by difference between the total reducing power and that remaining after treatment with glucose oxidase (16). The reducing power was measured by the method of Nelson (15); results showed that glucose was responsible for approximately 50% of the reducing power of the mixture. The remaining galactose in the mixture was identified by paper chromatography with the solvent of Marinetti, Ford, and Stotz (30). The results of these procedures, which were repeated with the other gangliosides, are shown in Table 1.

Hexosamine. The ratios of hexosamine to sphingosine for MG-2, MG-3, and MG-4 were all 1:1 (Table 1). Identification of N-acetylgalactosamine was done by paper chromatography and TLC. N-Acetylglucosamine and N-acetylgalactosamine were used as standards.

The sialic acids. Quantitative determinations of sialic acid and sphingosine indicated that MG-1 is a hematoside, that MG-2 and MG-3 are disialogangliosides, and that MG-4 is a trisialoganglioside (Table 1).

For identification purposes the sialic acid of MG-1 was split off by hydrolysis in 0.03 N HCl during 2 hr at 85°C; parallel experiments were run with gangliosides from rat brain and hematoside from horse red cells. The products of hydrolysis were partitioned with 1 ml of chloroform, and the aqueous phase was passed through a 2 × 8 cm column containing Dowex 1 (acetate form). After the column was washed with water, the sialic acid was eluted with 0.5 M sodium acetate. Free sialic acid was obtained by passing its salt through a column of Dowex 50 (H$^+$) (4). The solution of free acid was lyophilized and analyzed by TLC using n-propanol– 1 N ammonia–water 6:2:1 according to the method of Granzer (31). The plate was sprayed with the resorcinol reagent. In other experiments in which the sialic acid was chromatographed on paper with n-butanol–
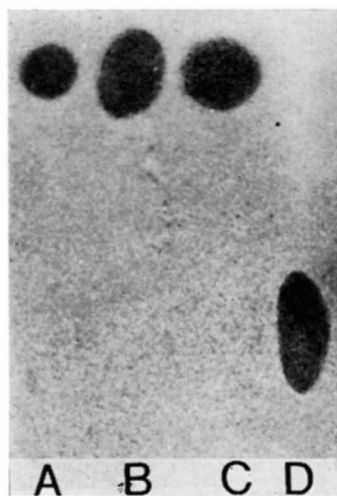
FIG. 2. Paper chromatography of sialic acid from rabbit muscle gangliosides. *A*, NANA; *B*, products of acid hydrolysis from gangliosides of rat brain; *C*, products of acid hydrolysis from gangliosides of rabbit muscle; *D*, NGNA. Solvent, *n*-butanol–*n*-propanol–1 N HCl 1:2:1; spray, thiobarbituric acid reagent (32).
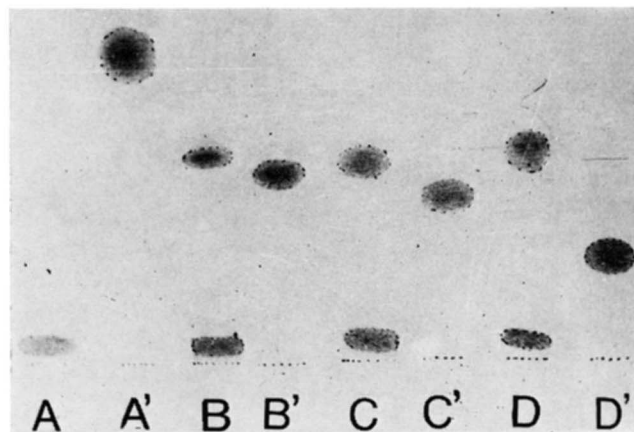

FIG. 3. Thin-layer chromatography of the products obtained from rabbit muscle gangliosides after treatment with neuraminidase from *Clostridium perfringens*. *A*, MG-1 after treatment with neuraminidase; *B*, MG-2 after treatment with neuraminidase; *C*, MG-3 after treatment with neuraminidase; *D*, MG-4 after treatment with neuraminidase; *A'*, MG-1; *B'*, MG-2; *C'*, MG-3; *D'*, MG-4. Solvent, CHCl₃–CH₃OH–10% NH₄OH 60:35:8; spray, resorcinol–HCl (23).

n-propanol-1 N HCl 1:2:1 as solvents, thiobarbituric acid reagent (32) was used to spray the paper (Fig. 2). In every case, NGNA isolated from hematoside from horse red cells and NANA from gangliosides from rat brain were used as standards. It was consistently found that the sialic acid of the gangliosides from rabbit muscle was *N*-acetylneuraminic acid; no indication of the presence of *N*-glycolylneuraminic acid or any other neuraminyl derivative was found. In agreement with the results of the chromatographic experiments, the reaction for the glycolyl group (12) was negative in the presence of muscle gangliosides as was that for the *O*-acetyl group by the method of Hestrin (13).

Treatment of individual gangliosides with neuraminidase

A sample of each gangliosidic fraction, containing approximately 60 μg of sialic acid, was dissolved in 0.15 ml of acetate buffer, pH 5.4. A neuraminidase solution containing 0.06 U of the enzyme from *Clostridium perfringens* in 0.1 ml was added, and the mixture was incubated at 37°C for 4 hr (33). The free sialic acid was separated by dialysis and identified by TLC as mentioned above. The contents inside the dialysis bag were analyzed by TLC. Using chloroform–methanol–10% ammonia 60:35:8 as solvent, the results showed that MG-1 had lost all of its sialic acid and that the remaining compound moved on TLC at the same level as a standard preparation of lactosylceramide obtained by partial hydrolysis of gangliosides from bovine brain. The compounds remaining after the enzymatic treatment of MG-2, MG-3, and MG-4 still contained part of their original sialic acid and, when subjected to TLC, they

moved approximately as a sample of monosialoganglioside (G_{M1}) standard isolated from rat brain (Fig. 3).

Fatty acids

Methyl esters of the fatty acids from muscle gangliosides were separated by chromatography on silica gel G plates developed with a mixture of *n*-hexane–ethyl ether 90:10 (34). Methyl esters of authentic samples of saturated, unsaturated, and hydroxy fatty acids were chromatographed simultaneously. The plates were sprayed with rhodamine 6G, and the spots were located under UV light (35).

The fatty acids were eluted successively with *n*-hexane and ether; the volumes of the solutions were reduced under N₂ and then analyzed by GLC. The samples in which hydroxy acids were investigated were previously silylated according to Penick and McCluer (36). Table 2 shows that all of the different gangliosides from muscle had similar fatty acid compositions. The most

TABLE 2. Fatty acids of the different types of gangliosides from rabbit muscle

Fatty Acid	MG-1	MG-2	MG-3	MG-4
	\% of total fatty acids			
14:0[a]		3.4	4.1	7.4
Unidentified		1.7	4.7	4.6
16:0	17.1	30.7	24.2	30.0
16:1	1.7	3.8	11.1	12.3
18:0	56.7	47.6	30.5	25.3
18:1	9.3	9.5	12.9	17.1
20:0	8.7	3.3	6.6	1.6
22:0	6.5		6.0	1.6

Fatty acids were analyzed by GLC as methyl esters on EGSS-X. The column was operated at 180°C.
[a] Number of carbon atoms:number of double bonds.

abundant fatty acids were 18:0 and 16:0, with significant amounts of 18:1, 16:1, 20:0, and 22:0. No hydroxy fatty acids were found.

Differences between MG-2 and MG-3

The structural differences between these two gangliosides are not readily apparent at the present time. The chromatographic relationships of these compounds were compared with the G_{D1a} of rat brain. It appeared that MG-3 ran on TLC similarly with G_{D1a} from brain, whereas MG-2 ran clearly in front of both. After treatment with neuraminidase, both MG-2 and MG-3 chromatographed as G_{M1}, suggesting that the difference between them was in the positions of the sialyl groups labile to the action of neuraminidase (Fig. 3).

The sphingosine moieties

Muscle gangliosides were isolated and purified until they appeared homogeneous in TLC with at least two solvents (see Materials and Methods). They were hydrolyzed for 18 hr at 70°C in a mixture of concentrated hydrochloric acid–water–methanol 8.6:9.4:82 (v/v/v), and the respective sphingosine moieties were isolated (26) and treated with metaperiodate according to Sweeley and Moscatelli (37); the aldehydes obtained were analyzed on a 1.30-m column of 3% SE-30 on Gas-Chrom CLA-DMCS.

The main aldehydes obtained after periodate oxidation of the sphingosines from the different muscle gangliosides were hexadecenal and octadecenal, thus showing that C_{18}-sphingenine and C_{20}-sphingenine are the main bases in these gangliosides as is the case for brain gangliosides. GLC of the products of oxidation of the sphingosine from MG-1 showed also the presence of small amounts of hexadecanal and octadecanal, showing that the C_{18}-sphinganine and C_{20}-sphinganine are also present (Table 3). To confirm the presence of C_{18}- and C_{20}-sphingenines, the aldehydes obtained by periodate oxidation were hydrogenated in ether solution by the use of palladium on charcoal (5%) as catalyst and under H_2 at a pressure of 1 kg/cm² (38). When the products of hydrogenation were analyzed by GLC, peaks corresponding to hexa- and octadecenal were displaced to the positions of hexadecanal and octadecanal.

An unidentified peak appeared in the product of metaperiodate oxidation of the sphingosine from each of the rabbit muscle gangliosides; the amounts in the various gangliosides were between 1.47 and 5.0% of the total aldehydes.

Possible contamination from red cell gangliosides

We considered the possibility that all or a significant part of the gangliosides obtained from muscle were, in a stricter sense, obtained from the red cells present in

TABLE 3. Long-chain base compositions of rabbit muscle gangliosides

LCB	Aldehyde	MG-1	MG-2	MG-3	MG-4
18-Sphinganine	Hexadecanal	3.6	tr[a]	tr	tr
18-Sphingenine	Hexadecenal	70.0	86.4	89.0	87.5
20-Sphinganine	Octadecanal	1.4			
20-Sphingenine	Octadecenal	20.0	10.8	8.7	11.0
Unidentified		5.0	2.8	2.3	1.5

LCB were analyzed by GLC on a 3% SE-30 column after oxidation to the aldehyde.

[a] tr, trace.

muscle. Quantitative determinations were made of the gangliosides from muscle and blood. From 130 ml of rabbit blood 1 g of stroma was obtained, which was extracted by the method of Trams and Lauter (20). The total amount of gangliosidic NANA in 100 ml of blood was of the order of 0.033 µmole. Assuming that 12 ml of blood is present in 100 g of muscle, and because the total amount of gangliosidic NANA in muscle is 5 µmoles/100 g, it was concluded that the contamination of muscle gangliosides by red cell gangliosides was not more than 0.1%.

DISCUSSION

The gangliosides found in rabbit muscle showed a chromatographic pattern similar to that of the gangliosides from organs other than those of the central nervous system (4, 5) in that the most abundant ganglioside is MG-1, followed by MG-3, MG-2, and MG-4. This similarity does not necessarily extend to the presence of either acetyl or glycolyl groups in the molecules or to the nature of the fatty acids. The gangliosides in the peripheral nervous system have been studied in the adrenal medulla (4), and their pattern also shows a prevalence of hematoside.

The concentration of gangliosides that we found in muscle is approximately 10-fold lower than the concentration found by Ledeen, Salsman, and Cabrera (4) in adrenal medulla. Since it is not impossible that nervous tissue is a constituent of muscle in a 10% proportion, the question of whether or not the gangliosides isolated from muscle come from the nervous tissue of muscle should be considered.

In this connection it should be mentioned that while Waser (39) found an increase of motor plates in mouse diaphragm after 7 days of denervation, Max et al. (3) found a substantial increase of hematoside in cat, rat, and rabbit medial gastrocnemii 8 days after cutting the sciatic nerve.

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